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# Quercetin, an Inhibitor of Heat Shock Protein Synthesis, Inhibits the Acquisition of Thermotolerance in a Human Colon Carcinoma Cell Line

Mototsugu Koishi,[1,2,3] Nobuko Hosokawa,[1] Mamoru Sato,[1] Akira Nakai,[1] Kazunori Hirayoshi,[1] Masahiro Hiraoka,[2] Mitsuyuki Abe[2] and Kazuhiro Nagata[1]

[1]*Department of Cell Biology, Chest Disease Research Institute, Kyoto University, 53 Shogoin-Kawaharacho, Sakyo-ku, Kyoto 606–01 and* [2]*Department of Radiology, Faculty of Medicine, Kyoto University, 54 Shogoin-Kawaharacho, Sakyo-ku, Kyoto 606*

**Here, we describe the effects of quercetin on the induction of thermotolerance as examined by colony forming assay in a cell line derived from human colon carcinoma (COLO320 DM). Cells became resistant to heat treatment at 45°C when they were preheated at 42°C for 1.5 h or at 45°C for 10 min. This induction of thermotolerance was almost completely inhibited by continuous treatment with 100 $\mu M$ quercetin during the first and second heating sessions, and the interval between. This effect of quercetin was demonstrated to be dose-dependent over a concentration range of 50–200 $\mu M$. Quercetin did not increase the thermosensitivity of non-tolerant cells. The presence of quercetin during the first conditioning heating was more effective in inhibiting thermotolerance than its presence during the second heating. Quercetin was also found to inhibit the acquisition of thermotolerance induced by sodium arsenite. Cycloheximide, a nonspecific inhibitor of protein synthesis, did not affect the acquisition of thermotolerance by the same cell line. Quercetin specifically inhibits the synthesis of all heat shock proteins so far reported previously, and this leads to inhibition of the induction of thermotolerance. Such inhibition of thermotolerance by quercetin may improve the efficacy of clinical fractionated hyperthermia.**

Key words: Quercetin — Thermotolerance — COLO320 DM cell line — Heat shock protein — Hyperthermia

Hyperthermia has been extensively studied biologically[1] and clinically[2] as a new and promising modality of cancer therapy. However, there are still some problems to be resolved in the clinical setting. One of the major problems is the induction of thermotolerance in tumors.[3] Because of this phenomenon, hyperthermia is usually performed only twice a week or less.[4] In addition, hyperthermic therapy alone has produced only marginally useful results.[2] A material which inhibits the induction of thermotolerance could contribute to improving the effects of fractionated hyperthermia.

When cells or organisms are exposed to heat shock, they respond by synthesizing a group of proteins called heat shock proteins (HSPs[4]).[1] HSPs are among the proteins most highly conserved during evolution, and they have been shown to have an important role, not only in stressed cells but also in cells under normal growth conditions. It is still uncertain just how HSPs participate the acquisition, maintenance, and decay of thermotolerance in stressed cells.[1,4,5] We have found that the bioflavonoid quercetin specifically inhibited the synthesis of HSPs, including HSP90, HSP70s, and HSP27. Quercetin also inhibited the synthesis of the newly found HSPs, HSP47[6,7] and HSP40.[8] However, quercetin did not inhibit the synthesis of other cellular proteins, as examined one-dimensional and two-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of [$^{35}$S]methionine-labeled cell extract. In order to clarify the role of HSPs in the acquisition of thermotolerance and the clinical usefulness of quercetin, we investigated the effects of this drug on the acquisition of thermotolerance by a human colon carcinoma cell line.

## MATERIALS AND METHODS

**Cell culture** COLO320 DM cells[9] were provided by the Japanese Cancer Research Resources Bank and were cultured in Dulbecco's modified Eagle's medium containing 10% fetal calf serum and 15 m$M$ $NaHCO_3$ at 37°C in a humidified 5% $CO_2$-95% air atmosphere. Subconfluent cell monolayers were trypsinized, plated at a cell density of $1.2\times10^5$/ml in 25 cm$^2$ glass flasks, and incubated at 37°C for 24–48 h prior to use.

**Hyperthermia** Heat treatment was administered by immersing the flasks (Pyrex glass, 25 cm$^2$, Tokyo) in a water bath (KT35D, Advantec Toyo Co., Tokyo). The accuracy of thermoregulation was within 0.05°C. During heating, the pH of the medium was maintained at 7.2–7.4 by fastening the caps of the flasks to keep the concentra-

[3] To whom correspondence should be addressed. Present address: Department of Radiology, Faculty of Medicine, Kyoto University, 54 Shogoin-Kawaharacho, Sakyo-ku, Kyoto 606.

[4] The abbreviations used are: HSPs, heat shock proteins; DMSO, dimethyl sulfoxide; TCA, trichloroacetic acid.

tion of $CO_2$ constant. These flasks were then transferred to a water bath at 37°C for 3 min. After treatment, the cells were prepared for the colony-forming assay.

**Treatment with quercetin, cycloheximide, and sodium arsenite** These reagents were purchased from Nacalai Tesque (Kyoto). Quercetin was dissolved in dimethyl sulfoxide (DMSO, Nacalai Tesque), and was stored at a concentration of 50 m*M*. We added quercetin (50–200 $\mu M$) to the medium for 1 h before the scheduled heating time for preincubation. Cycloheximide was dissolved in minimal essential medium, stored at a concentration of 5 mg/ml, and used at a concentration of 5 $\mu$g/ml in a similar way to quercetin. Sodium arsenite was dissolved in minimal essential medium, and was stored at a concentration of 50 m*M*. Cells were treated with 200 $\mu M$ sodium arsenite at 37°C for 1 h, rinsed, and then incubated at 37°C for 6 h before undergoing a heat challenge at 45°C.

**Colony-forming assay** After each treatment, cells were trypsinized, counted with a hemocytometer, and plated at appropriate dilutions. Colonies were stained and were counted after 7–10 days of incubation at 37°C. Dishes containing 100–200 colonies were used for the calculation of survival. Plating efficiency was 70–90%. Data points given represent the mean of at least 3 plates, and the standard deviation is also given for each survival point.

**Metabolic label and SDS-PAGE** Cells were labeled with 100 $\mu$Ci of [$^{35}$S]methionine (specific activity, >1,000 Ci/mmol) in 1 ml of methionine-free minimal essential medium for 1 h at 37°C. After washing with cold phosphate-buffered saline (PBS), the cells were lysed in a buffer containing 1% Nonidet P-40, 0.15 m*M* NaCl, 50 m*M* Tris-HCl (pH 8.0), 5 m*M* EDTA and protein inhibitors (2 m*M* phenylmethylsulfonyl fluoride, 2 m*M* N-ethylmaleimide, 1 $\mu$g/ml pepstatin and 1 $\mu$g/ml leupeptin). The cell lysates prepared from equal numbers of the cells were centrifuged at 12,000*g* for 20 min, and analyzed by one-dimensional SDS-PAGE according to Laemmli's method.[10] The molecular weight markers (phosphorylase b, 94 kDa; albumin, 67 kDa; ovalbumin, 43 kDa; carbonic anhydrase, 30 kDa; trypsin inhibitor, 20 kDa; $\alpha$-lactalbumin, 14.4 kDa) were purchased from Pharmacia LKB.

## RESULTS

To induce thermotolerance, we treated COLO320 DM cells at 45°C for 10 min and then incubated them at 37°C for 6 h to allow recovery. The acquisition of thermotolerance was examined by determining the slope of the curves of surviving fraction of the cells versus treatment period of the second heat shock at 45°C, as shown in Fig. 1. The cells gained a $10^3$-fold greater resistance to the subsequent heat shock (45°C for 1 h) as measured by colony-forming ability in comparison to non-tolerant cells (Fig. 1). The difference of regression coefficients of tolerant and non-tolerant cells calculated by the method of least squares was statistically significant ($P<0.005$). Quercetin inhibited the induction of thermotolerance at a concentration of 100 $\mu M$ when it was added to the culture from 1 h before the first conditioning heat treatment to the end of the second heating. After heating at 45°C for 30 min, the colony-forming ability of cells treated with quercetin was reduced to $10^{-3}$ of the control level (without quercetin treatment). The difference of regression coefficients of cells treated with and without quercetin was also statistically significant ($P<0.005$). Treatment with quercetin did not affect the slope of the survival curve for a single heat treatment at 45°C. The difference of regression coefficients of single-heated cells with and without quercetin was not statistically significant.

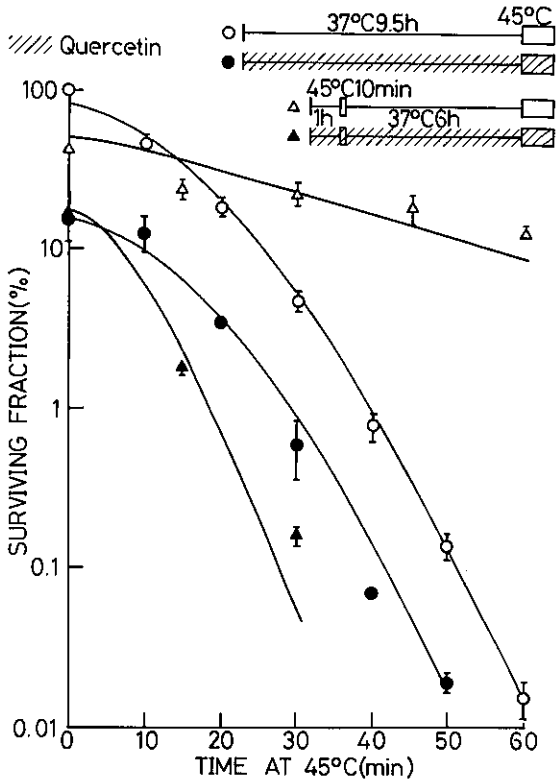


Fig. 1. Effects of quercetin on the surviving fraction of thermotolerant or non-tolerant cells as a function of the heating time at 45°C. Cells were preincubated with (●) or without (○) 100 $\mu M$ quercetin for 9.5 h prior to heat treatment at 45°C. Cells were incubated with (▲) or without (△) quercetin from 1 h prior to the conditioning heat treatment (45°C, 10 min) to the end of the challenging heat treatment (45°C) and then during the recovery period (37°C for 6 h).

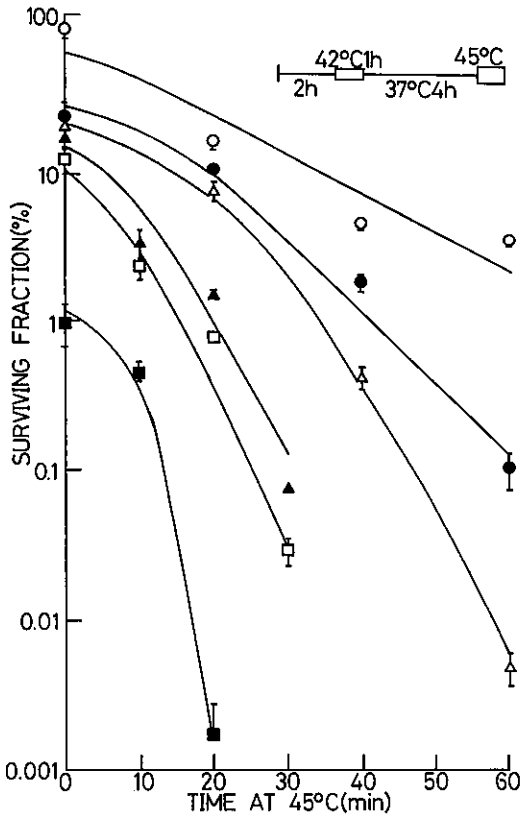


Fig. 2. Dose-dependent effect of quercetin on the time-survival curves of thermotolerant cells after hyperthermic treatment. Cells were incubated with quercetin for 2 h prior to the conditioning heat treatment (42°C, 1 h), for 4 h during recovery at 37°C and for the indicated periods of 45°C heat challenge at the concentrations of 0 $\mu M$ (○), 50 $\mu M$ (●), 75 $\mu M$ (△), 100 $\mu M$ (▲), 150 $\mu M$ (□), and 200 $\mu M$ (■).

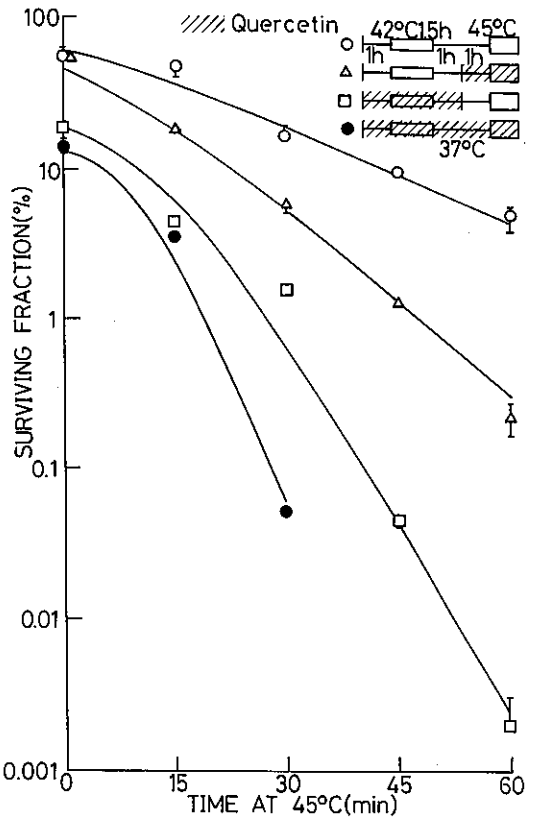


Fig. 3. Effects of quercetin on the first conditioning heat treatment and the second lethal heat treatment. For the first conditioning treatment, we added 100 $\mu M$ quercetin to the medium 1 h prior to heating at 42°C for 1.5 h. Cells were rinsed 1 h later, allowed to recover by 37°C incubation for 1 h, and then treated at 45°C (□). For the second lethal heat treatment, after the first conditioning treatment and recovery at 37°C for 1 h, we added quercetin to the medium from 1 h before the second lethal heat to the end of it (△). The effect of incubation with (●) or without (○) quercetin throughout the whole period is also shown in the figure.

Dose-dependence of the effect of quercetin was examined over the range of concentrations between 50 and 200 $\mu M$. Cells were heated at 42°C for 1 h followed by incubation at 37°C for 6 h, and then heat-challenged at 45°C. Quercetin was added to the culture 2 h before the first conditioning heating as above. The changes in the slopes of the survival curves following heating at 45°C showed that quercetin dose-dependently inhibited the acquisition of thermotolerance (Fig. 2).

To examine the period of incubation required for quercetin to inhibit the induction of thermotolerance, we added quercetin to the medium for various periods at different time points (Fig. 3). When quercetin was added to the cultures 1 h prior to the first heating (42°C for 1.5 h) and then removed by washing the culture with medium 1 h after the end of the first heat treatment, it efficiently inhibited the acquisition of thermotolerance (open squares in Fig. 3). On the other hand, the presence of quercetin only 1 h prior to and during the second heat treatment at 45°C was less effective (open triangles in Fig. 3). The effect of quercetin was strongest when it was present during the whole culture period (closed circles in Fig. 3).

Since quercetin is reported to inhibit protein synthesis through the inhibition of RNA polymerase II, we examined the effect of cycloheximide on the inhibition of thermotolerance induction. Although quercetin decreased general protein synthesis to 60–80% of the control level at a concentration of 100 $\mu M$, it completely inhibited the induction of HSPs, HSP70s and HSP90

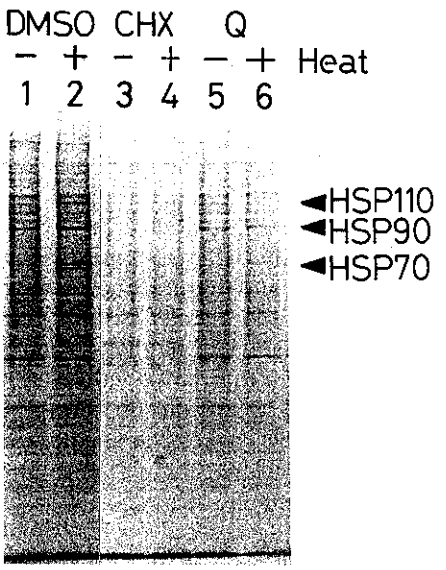


Fig. 4. One-dimensional gel electrophoresis of COLO320 DM cells. To examine the effects of quercetin and cycloheximide on the protein synthesis, cells were pretreated with 100 $\mu M$ quercetin (lanes 5 and 6) or 5 $\mu$g/ml cycloheximide (lanes 3 and 4) or 0.25% DMSO for 2 h, and heat shocked at 43°C (lanes 2, 4, and 6) for 1.5 h, or maintained at 37°C (lanes 1, 3, and 5). After incubation at 37°C for 2 h, cells were labeled with 0.1 mCi of [$^{35}$S]methionine for 1 h.

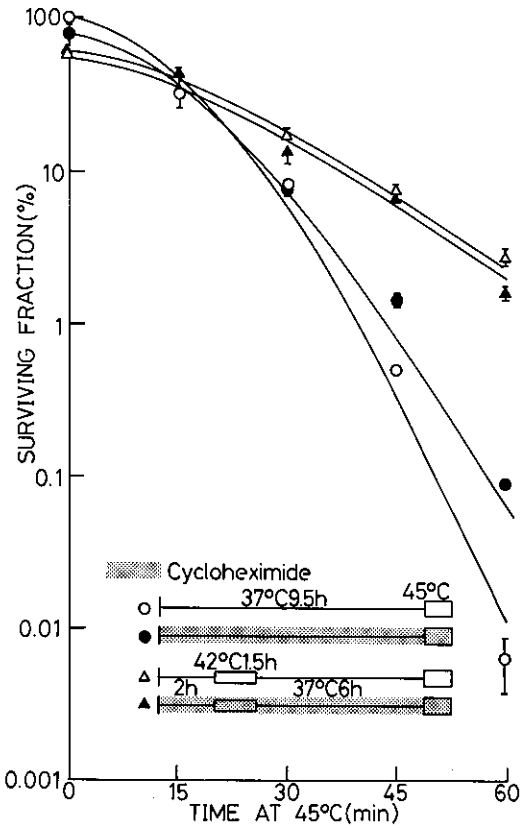


Fig. 5. Effects of cycloheximide on the induction of thermotolerance. Cells without preheating at 42°C for 1.5 h in the presence (●) or absence (○) of cycloheximide (5 $\mu$g/ml), and those with the preheating in the presence (▲) or absence (△) of cycloheximide were heated at 45°C for the indicated period.

after heat shock, as detected by one-dimensional SDS-PAGE (Fig. 4). We added cycloheximide to the cultures in the same way as quercetin. Cycloheximide inhibited up to 90% of the control level of general protein synthesis at a concentration of 5 $\mu$g/ml when measured in terms of the incorporation of [$^{35}$S]methionine into the TCA-insoluble fraction of the cells. Cycloheximide markedly reduced the general protein synthesis without affecting the incorporation of [$^{35}$S]methionine into particular bands (Fig. 4). The addition of cycloheximide to the medium did not prevent the acquisition of thermotolerance (Fig. 5). Cell survival was slightly enhanced when cells were exposed to a single heating at 45°C in the presence of cycloheximide. These results suggested that the inhibition of acquired thermotolerance by quercetin was due to specific inhibition of the synthesis of HSPs rather than general inhibition of protein synthesis.

When cells were treated with 200 $\mu M$ sodium arsenite at 37°C for 1 h, rinsed, and incubated at 37°C for 6 h before a 45°C heat challenge, they also acquired thermotolerance (Fig. 6). However, cells failed to become thermotolerant when they were incubated with quercetin during the whole period of sodium arsenite treatment (Fig. 6).

## DISCUSSION

Quercetin is a bioflavonoid which exists widely in plants including many kinds of fruit and vegetables.[11] Among a number of biological effects,[12–16] quercetin is reported to be a hyperthermic sensitizer for HeLa cells when added during a single heating at 41–42°C.[14] Kim *et al.* suggested that this sensitization is due to a decrease in intracellular pH resulting from the inhibition of lactate transport. The present study demonstrated that quercetin probably inhibits the acquisition of thermotolerance via the inhibition of HSPs synthesis. Heat sensitization by quercetin was not observed in this study. The heat sensitization noted by Kim *et al.* might therefore be partly explained by inhibition of the acquisition of thermotolerance by this agent, because thermotolerance is known to be induced during heating at temperatures of 41–42°C.[4] The reason why the cells were not sensitized to heat at

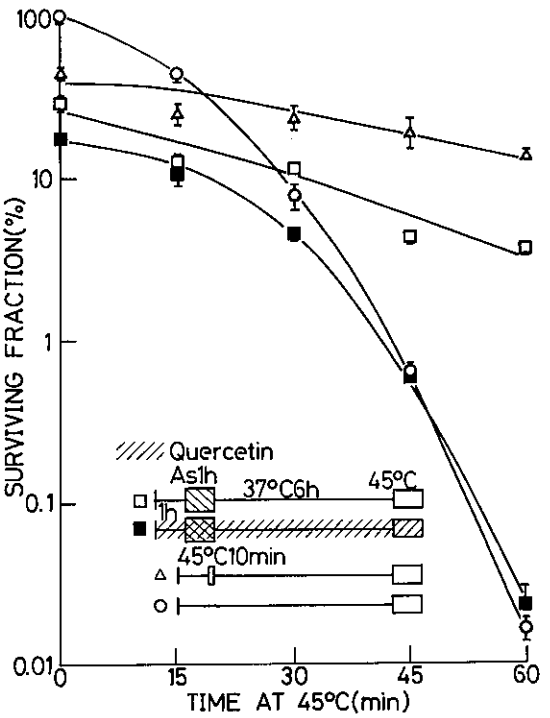


Fig. 6. Effects of quercetin on the thermotolerance induced by addition of sodium arsenite. Cells acquired thermotolerance when treated with 200 $\mu M$ sodium arsenite for 1 h, rinsed, and incubated at 37°C for 6 h (□). Preincubation with 100 $\mu M$ quercetin from 1 h prior to sodium arsenite treatment to the end of the 45°C heat challenge reduced thermotolerance (■). Non-tolerant cells (○) and cells with thermotolerance acquired by heating at 45°C for 10 min (△) are also shown in the figure. As: sodium arsenite.

45°C is that this temperature might cause severe cellular damage which exceeds the protective capacity of HSPs, so that inhibition of the synthesis of HSPs would not be critical for cell killing at such a high temperature.

Cycloheximide had no effect on the acquisition of thermotolerance in our system (Fig. 5), which suggested that the inhibitory effect of quercetin on the acquisition of thermotolerance is not due to the nonspecific inhibition of protein synthesis, but to the specific inhibition of induction of HSPs. There are conflicting reports concerning the effects of cycloheximide on the induction and/or inhibition of acquired thermotolerance. Some reported that cycloheximide had no effect on the acquisition of thermotolerance in Chinese hamster ovary cells,[17-19] and another claimed that it actually inhibited the acquisition of thermotolerance.[20] It seems contradictory that cycloheximide does not inhibit the induction of thermotolerance, because it inhibits the total protein synthesis including HSPs. However, polypeptides during *de novo* synthesis are most sensitive to heat treatment, because the folding and/or assembly of the subunits are not completed. Cycloheximide inhibits the total protein synthesis, and these quiescent cells therefore contain few heat-labile immature polypeptides, and so should be resistant to treatment at high temperature, even if the synthesis of HSPs is inhibited by this agent.

The treatment of the cells with quercetin is reported to cause perturbation of the cell cycle, and the major effect is to freeze the cell cycle pattern at the concentration of quercetin used in this experiment.[21] Although heat sensitivity of the cells is known to be dependent on the cell cycle, the possibility can thus be excluded that quercetin arrested the cells at a heat-resistant phase of the cell cycle, which might have caused the apparent heat resistance. We also used cycloheximide and sodium arsenite in this study. Cycloheximide was reported to inhibit cell proliferation by freezing the cell cycle, not by causing arrest in a specific phase.[22] It is not known whether sodium arsenite induces a specific blockage of the cell cycle.

We have already reported that quercetin and other flavonoids including genistein, flavone, luteolin, and kaempferol inhibit the synthesis of HSP110, HSP90, HSP70s, HSP47, HSP40, and HSP27 without affecting the synthesis of other cellular proteins.[20] Northern blot analysis using the probe for HSP70 established that this inhibition is at the level of mRNA accumulation. We have recently observed that quercetin inhibits the transcription of HSP70 mRNA through inhibition of the activation of heat shock factor (HSF) in a study using RNase protection assay, CAT analysis, and gel shift analysis.[23] Quercetin also inhibited HSF activation *in vitro*; this was established by treating the cell extract at elevated temperature or with urea as described by Mosser *et al.*[24] While quercetin completely inhibited the induction of HSPs, it did not decrease general protein synthesis by more than 20–40% of the control level.[20] Since quercetin was shown not to inhibit the binding of other DNA-binding proteins such as octamer-binding proteins to the *cis*-responsive element, octamer-binding motif,[23] the inhibitory effect of quercetin is concluded to be specific for the synthesis of HSPs.

It is known that the effect of quercetin is partially blocked when the growth medium contains a high concentration of bicarbonate.[16] Both inhibition of the synthesis of HSPs and inhibition of the acquisition of thermotolerance by quercetin were subject to interference by the presence of $>33$ m$M$ bicarbonate (data not shown). This also suggests a correlation between the synthesis of HSPs and the induction of thermotolerance.

The action of quercetin during the first conditioning heat treatment and during the second heating, in terms of its effect on the induction of thermotolerance, was

examined (Fig. 3). Quercetin was more effective when added during the first conditioning heating than the second heating session, suggesting that the induction of HSPs after the first heating is important in the acquisition of thermotolerance. Nevertheless, the possibility remains that there are other mechanisms apart from HSPs which are involved in the acquisition of thermotolerance.

Induction of thermotolerance by sodium arsenite treatment was also inhibited by quercetin, which is consistent with previous reports.[18, 20, 25] These results also indicate the contribution of HSPs to the acquisition of thermotolerance.

There are several reports which have suggested that HSPs are responsible for the acquisition of thermotolerance,[25-34] but others take a different standpoint.[17-19, 26] Recent studies in mammalian cells have directly indicated an important role of HSPs in the induction of thermotolerance.[35-39] For example, inactivation of HSP70 by the microinjection of a specific antibody or the reduction of its expression by genetic means causes cells to become thermosensitive.[35-37] In contrast, transfection of the HSP27 or HSP70 genes increases thermoresistance.[38, 39] However, which HSPs are actually responsible for thermotolerance remains to be determined.[40] Since quercetin inhibits the induction of all the major HSPs reported to date and has a relatively low toxicity *in vivo*,[41] it would seem possible to use it as a heat-sensitizer in hyperthermic cancer therapy. Thus, further studies on quercetin and other flavonoids *in vivo* would appear to be worthwhile.

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