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Flavonoids Inhibit the Expression of Heat Shock Proteins

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ABSTRACT. **Cells exposed to several forms of stress, such as heat shock, transiently synthesize a group of proteins called heat shock proteins (hsps). Although many stressors other than heat shock are known to induce hsps, inhibitors of hsp expression have never been reported. Here we show that quercetin and several other flavonoids inhibit the synthesis of hsps induced by heat shock in two human cell lines, Hela cells and COLO320 DM cells. Quercetin inhibited the induction of hsp70 at the level of mRNA accumulation. This is the first report to describe the inhibition of hsp expression by reagents.**

When cells or organisms are exposed to heat shock, they respond by synthesizing a group of proteins called heat shock proteins (hsps) (19). Hsps are among the most highly conserved proteins during the evolution, and the important roles of hsps have been clarified, not only in stressed cells but also in cells under normal growth conditions. For example, some members of the hsp70 family are reportedly involved in protein translocation into the mitochondria and endoplasmic reticulum (ER) (6, 7), and mitochondrial hsp60 in yeast is shown to be essential for protein assembly (4, 31). Grp78 is well known to bind to immunoglobulin heavy chains and prevent their aggregation in the ER (23). Hsp90 binds to glucocorticoid receptors or $pp60^{src}$ to regulate their activity and translocation through interaction with the cytoskeleton (30, 33). The induction of hsps is observed under various adverse circumstances other than heat shock, such as hypoxia, glucose starvation, and the addition of sodium arsenite, heavy metals or amino acid analogues, but materials which specifically inhibit the induction of hsps have never been reported.

Quercetin is a bioflavonoid, which is distributed widely in plants including many kinds of fruits and vegetables (15). Although it is known to have mutagenicity (3) and genotoxicity (20), it lacks carcinogenicity *in vivo* (2). A number of biological effects of quercetin have been reported; the inhibition of cultured cell growth (12, 36), inhibitory effects on glycolysis (36), macromolecule synthesis (10), activity of protein kinases (11), and ATPases (13). Other flavonoids also have similar activities to different degrees. Quercetin is reported to be a hyperthermic sensitizer in HeLa cells (12).

Here we report that quercetin and several other flavonoids inhibit the synthesis of hsps including hsp90, hsp70s, hsp47 and hsp28 induced by heat shock, sodium arsenite, and L-azetidine 2-carboxylic acid (Azc) in two human cell lines, namely HeLa cells and COLO320 DM cells derived from a human colon cancer (34).

MATERIALS AND METHODS

Materials. Quercetin and rutin were purchased from Nacalai Tesque (Kyoto, Japan), and kaempferol was obtained from Sigma. Genistain, flavone and luteolin were from Extrasynthèse (Genay, France). These flavonoids were dissolved in dimethyl sulfoxide (DMSO), purchased from Nacalai Tesque, and stored at a concentration of 20–120 mM. Sodium arsenite and Azc were obtained from Nacalai Tesque, leupeptin and pepstatin were from Peptide Institute (Osaka, Japan). Ampholine carrier ampholites (pH 3.5–10.0), Protein A-Sepharose and Protein-G Sepharose were purchased from Pharmacia LKB (Uppsala, Sweden). L-[^{35}S]methionine (>37

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Abbreviations used: hsp, heat shock protein; ER, endoplasmic reticulum: Azc, L-azetidine 2-carboxylic acid; DMSO, dimethyl sulfoxide; FBS, fetal bovine serum; MEM, Eagle's minimal essential medium; PBS, phosphate-buffered saline; TCA, trichloroacetic acid; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; hsc, heat shock cognate; grp, glucose-regulated protein; HSE, heat shock element; HSF, heat shock factor; CAT, chloramphenicol acetyl transferase.

TBq/mmol) and L-[4,5-^{3}H]leucine (4.4–7.0 TBq/mmol) were from Amersham Japan (Tokyo, Japan), and [α-^{32}P]dCTP (111 TBq/mmol) was purchased from New England Nuclear (Boston, MA).

Antibodies and Probes. Rabbit anti-mouse hsp90 antiserum was kindly provided by Dr. I. Yahara (The Tokyo Metropolitan Institute of Medical Science), and rabbit anti-mouse hsp70 antiserum was a kind gift from Dr. K. Ohtsuka (Aichi Cancer Center Research Institute). 7C8B1 is a rat monoclonal antibody to anti-chick hsp47 (35), and rabbit anti-human hsp28 antibody was a generous gift from Dr. W. J. Welch (University of California, San Francisco). The probe used to detect mRNA of hsp70 was pH 2.3 (40), which was kindly provided from Dr. R. I. Morimoto (Northwestern University, Chicago).

Cell Culture. COLO320 DM cells were derived from human colon cancer, and provided by the Japanese Cancer Research Resources Bank. COLO320 DM and HeLa cells were cultured in Dulbecco's modified Eagle's minimal essential medium (Nissui Pharmaceutical, Tokyo, Japan), supplemented with 10% fetal bovine serum (FBS) (Flow, Sidney, Australia) at 37°C in a 5% CO_2 atmosphere.

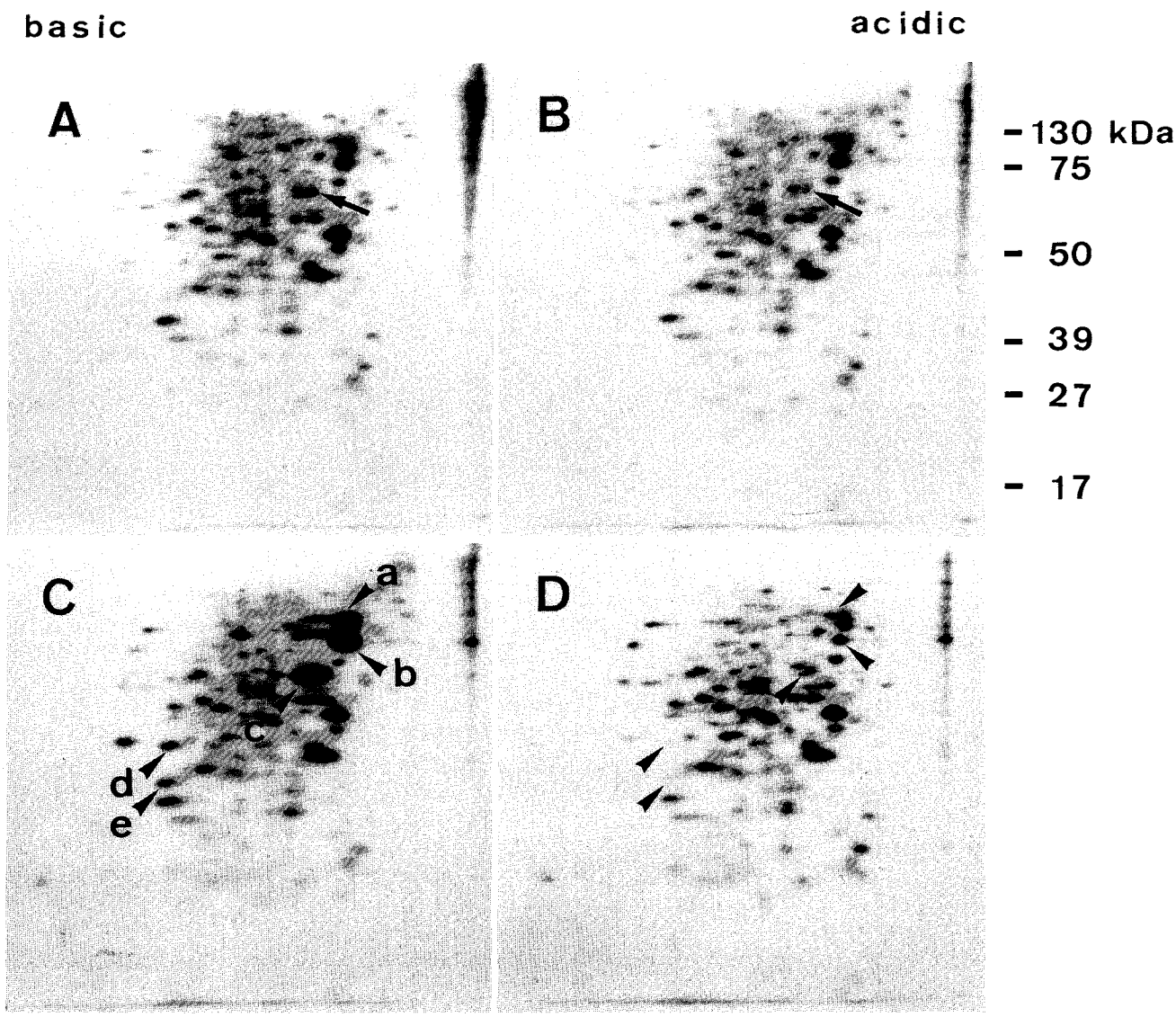

Fig. 1. Two-dimensional gel electrophoresis of COLO320 DM cells. Cells were pretreated with 100 μM quercetin or 0.25% DMSO for 6 h, followed by heat shock at 43°C for 1.5 h. After incubation at 37°C for 2 h, cells were labeled with [^{35}S]methionine for 1 h. Quercetin or DMSO as a vehicle was present during heat shock and recovery as well as the pretreatment period. A, Vehicle (0.25% DMSO) without heat treatment; B, 100 μM quercetin without heat treatment; C, vehicle with heat shock; D, 100 μM quercetin with heat shock. Arrows indicate the hsc70 (p72), and arrowheads show inducible-type hsps (a: hsp110, b: hsp90, c: hsp70, d: hsp47, e: hsp40).

Drug addition, Heat-shock and Metabolic Labeling. About 1×10^6 cells in 35 mm-diameter plastic dish (Nunc, Roskilde, Denmark) inoculated two days before were incubated in the presence of flavonoids for 6 h prior to heat shock. COLO320 DM cells were heat shocked at 43°C for 1.5 h, and HeLa cells, for 1 h. As a control, cells were incubated at 37°C instead of at 43°C for the same period. In all the experiments in this report, flavonoids or 0.25% DMSO as the vehicle, were present in the medium from 6 h prior to heat shock, during heat shock and at the end of the 2 h recovery period, except in one experiment in which the effect of quercetin addition at each period was examined. For metabolic labeling, cells were washed with Dulbecco's phosphate-buffered saline (PBS) without Ca^{2+} nor Mg^{2+}, and incubated for 1 h with 925 kBq of [^{35}S]methionine in 0.5 ml of methionine-free Eagle's minimum essential medium (MEM) supplemented with 10% dialysed FBS. To detect hsp28, HeLa cells were labeled with [^{3}H]leucine for 1 h in 0.5 ml of leucine-free MEM containing 10% dialysed FBS.

The final concentration of each flavonoid added to the medium was determined so that the cell number did not increase in the presence of the drug during three day's incubation.

To examine the effect of quercetin on the induction of hsps by sodium arsenite, cells were exposed to sodium arsenite at a final concentration of 50 μM for 1 h, recovered for 2 h without sodium arsenite, then labeled with [^{35}S]methionine for 1 h. Quercetin or vehicle (DMSO) was present in the medium from 6 h prior to the treatment of sodium arsenite to the end of the recovery period. In the case of Azc, cells were incubated with 5 mM (HeLa cells) or 10 mM (COLO320 DM cells) of Azc for 12 h before labeling with radioactive methionine. Quercetin or vehicle was present in the medium simultaneously with Azc.

Preparation of Cell Lysates. After metabolic labeling, cells were washed twice with PBS, and lysed in buffer containing 1% Nonidet P-40, 0.15 mM NaCl, 50 mM Tris-HCl (pH 8.0), 5 mM EDTA and protease inhibitors (2 mM phenylmethylsulfonyl fluoride, 2 mM N-ethylmaleimide, 1 μg/ml pepstatin and 1 μg/ml leupeptin) (14). The supernatant centrifuged at 12,000 × g for 20 min was analysed.

Gel Electrophoresis. Cell extracts containing equal amounts of trichloroacetic acid (TCA)-insoluble radioactivity were analyzed by one-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according to

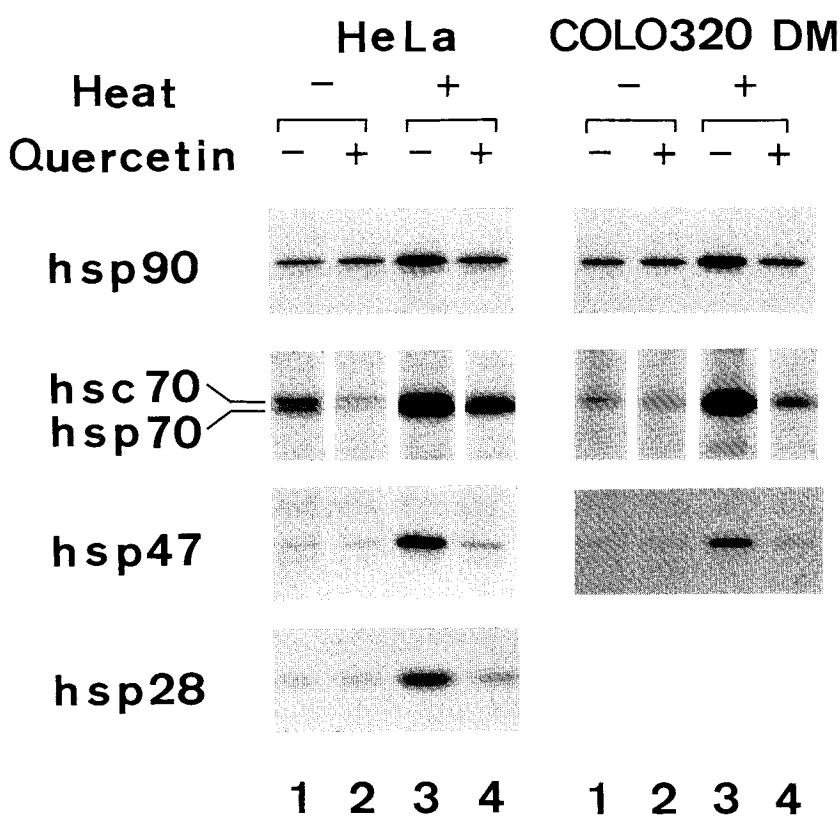

Fig. 2. Immunoprecipitation of HeLa and COLO320 DM cells using specific antibodies. Cells treated with or without heat shock in the presence or absence of quercetin were labeled with [^{35}S]methionine, and the preparation of cell extract and the immunoprecipitation were performed as described under materials and methods. The specific antibodies used were anti-hsp90, anti-hsp70, anti-hsp47, and anti-hsp28. HeLa and COLO320 DM cells were heat-shocked at 43°C for 1 and 1.5 h, respectively, and recovered at 37°C for 2 h. Quercetin was added 6 h before heat shock to a final concentration of 50 μM and 100 μM for HeLa and COLO320 DM cells, respectively.

the methods of Laemmli (16), or two-dimensional gel electrophoresis (first dimension was a non-equilibrium pH gradient gel electrophoresis and the second was 10% SDS-PAGE) (28). The molecular weight markers used for the one-dimensional SDS-PAGE were purchased from Pharmacia LKB (phosphorylase b, 94 kDa; albumin, 67 kDa; ovalbumin, 43 kDa; carbonic anhydrase, 30 kDa; trypsin inhibitor, 20 kDa; α-lactalbumin, 14.4 kDa), and pre-stained molecular weight markers for two-dimensional gel electrophoresis were obtained from BIORAD (phosphorylase b, 130 kDa; bovine serum albumin, 75 kDa; ovalbumin, 50 kDa; carbonic anhydrase, 39 kDa; soybean trypsin inhibitor, 27 kDa; lysozyme, 17 kDa).

Immunoprecipitation. Cell lysates containing equal amounts of TCA-insoluble radioactivity were incubated for 30 min at 4°C with each sepcific antibody or preimmune serum. Antigen-antibody complexes adsorbed to Protein A-Sepharose were eluted by boiling in Laemmli's electrophoresis sample buffer, and analysed by 10% SDS-PAGE (24). Protein G-Sepharose was used to precipitate the immunocomplex of hsp47 with rat monoclonal antibody against hsp47.

Northern Blot Analysis. On day 2 of inoculation, approximately 5×10^6 cells per 10 cm-diameter dish were incubated with or without quercetin, and heat treated as described above. Equal amounts of total RNA isolated by the quanidium/cesium chloride method (5) were electrophoresed in formaldehyde-containing agarose gels, transferred to a nylon membrane, and hybridized with [α-^{32}P]dCTP-labeled probes for hsp70 or β-actin, as an internal control.

RESULTS

Inhibition of hsp synthesis by quercetin, analysed by two-dimensional gel electrophoresis or by immunoprecipitation. COLO320 DM cells were labeled with [^{35}S]-methionine for 1 h, and protein synthesis analyzed by two-dimensional gel electrophoresis as described in materials and methods. Figure 1 shows the profiles of cellular proteins synthesized in COLO320 DM cells with or without heat shock treatment. As reported in human cells (37), two members of the hsp70 family, hsc70 (p72) and hsp70, were synthesized constitutively at the normal temperature (Fig. 1a). Quercetin slightly inhibited this constitutive synthesis of both hsc70 and hsp70 (Fig. 1b). When cells were heat shocked, major hsps, such as hsp110, hsp90, and hsp70 were drastically induced (Fig. 1c). Hsp47, which we previously reported as being a collagen-binding transformation-sensitive hsp (25, 26, 27), and a novel basal hsp, the molecular size of which is 40,000 daltons (29), were also induced (Fig. 1c). Adding 100 μM quercetin to the cells specifically inhibited the

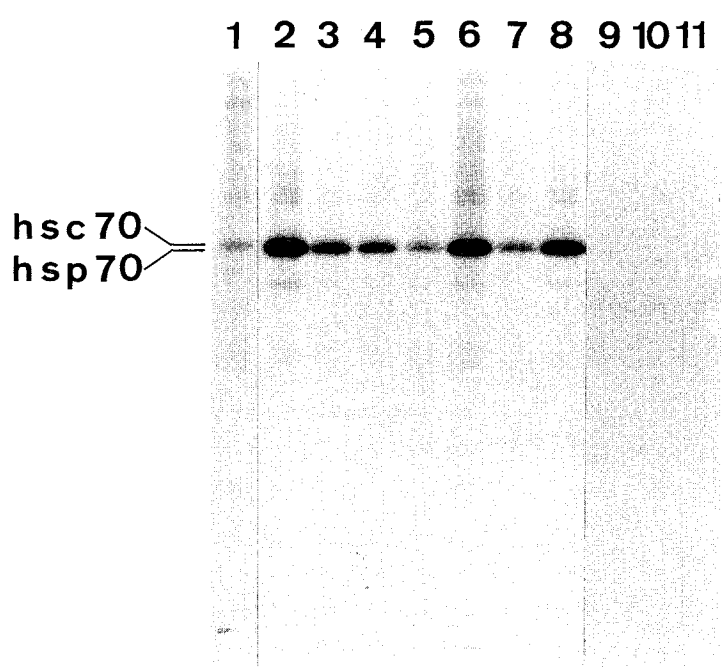

Fig. 3. Dose-dependent inhibition of hsp70 synthesis by quercetin (lanes 2–5) and the effect of the period of quercetin treatment on the hsp70 induction (lane 6–8) in COLO320 DM cells. Lane 1, without heat treatment; lanes 2–8, with heat treatment. Lanes 1 and 2, vehicle (0.25% DMSO); lane 3, 30 μM quercetin; lane 4, 60 μM quercetin; lanes 5–8, 100 μM quercetin. Periods of quercetin treatment were: lanes 3–5, before (6 h) and during (1.5 h) heat shock, and during the recovery period after heat shock (2 h); lane 6, pretreatment only; lane 7, only during the heat shock; and lane 8, only during the 2 h recovery period after heat shock. Lanes 9–11 were the same samples as lanes 1–3, precipitated with preimmune serum. Heat shock, metabolic labeling, cell preparation, and immunoprecipitation using anti-hsp70 antibody were performed as described in materials and methods. To examine the period of quercetin treatment, cells were washed twice with 2 ml of PBS to remove quercetin.

expression of all these hsps (shown by arrowheads in Fig. 1d), while the synthesis of other proteins remained essentially the same. Similar results were obtained using HeLa cells (data not shown).

The specific inhibition of the synthesis of three hsps, including hsp90, hsp70s (hsc70 and hsp70), and hsp47, by quercetin was confirmed in HeLa cells as well as in COLO320 DM cells by immunoprecipitation with specific antibodies. As mammalian hsp28 lacks methionine (38), HeLa cells were labeled with [^{3}H]leucine for the immunoprecipitation of hsp28 with the anti-hsp28 antibody. The synthesis of hsp70 and hsc70 at the normal temperature was slightly inhibited by quercetin, whereas that of hsp90, hsp47, and hsp28 was hardly affected (Fig. 2, lane 2). When the cells were treated at 43°C for 1 h (HeLa cells) or for 1.5 h (COLO320 DM cells), the induction of all four hsps was clearly observed (Fig. 2, lane 3). In the presence of quercetin, the induction of these hsps was inhibited to the level without heat shock (Fig. 2, lane 4). Partial inhibition of hsp70 synthesis was observed in HeLa cells. Preimmune serum did not precipitate any detectable band corresponding to that of each antigen (data not shown, see Fig. 3, lanes 9–11 for non-specific bands precipitated by preimmune rabbit serum).

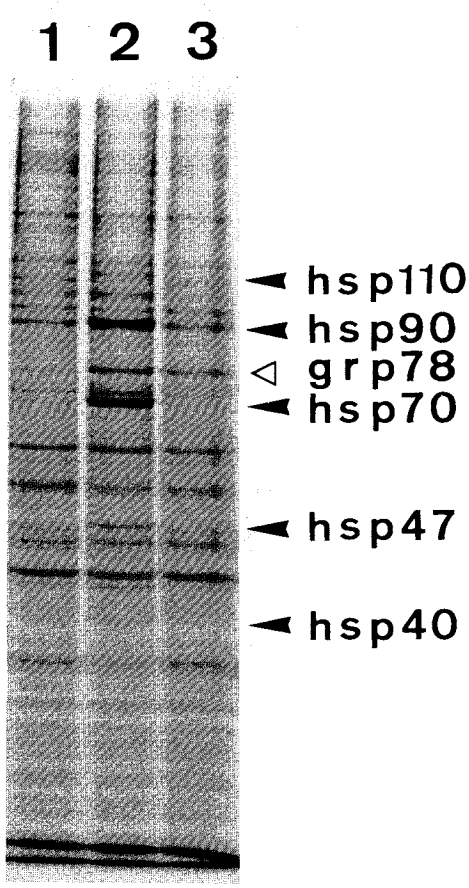

Fig. 4. Inhibition of hsp synthesis induced by Azc. HeLa cells were treated with 5 mM Azc for 12 h. To examine the effect of quercetin on the induction of hsps, 50 μM of quercetin or vehicle were added for the same period. Lane 1, control (without Azc nor quercetin); lane 2, Azc-treated cells without quercetin; lane 3, co-treatment of Azc and quercetin. Arrowheads indicate induced hsps, and an open triangle shows grp78.

Dose response and the effect of the period of quercetin treatment. The inhibition of hsp70 induction was examined at three different quercetin concentrations (30, 60 and 100 μM), and dose-dependent inhibition of hsp70 synthesis was observed by immunoprecipitation using anti-hsp70 antibody (Fig. 3, lanes 2–5). In the above experiments, quercetin was present from 6 h before heat shock to the end of the recovery period. We then examined the effect of the period of quercetin treatment on the inhibition of hsp70 synthesis. When quercetin was present only during heat stress, inhibition was at a similar level to that when it was present throughout the entire period (Fig. 3, compare lane 7 to lane 5). Treatment of cells with quercetin only before or only after heat shock caused weaker inhibition of hsp70 synthesis (Fig. 3, lanes 6 and 8). The observation that pretreatment with quercetin caused a weaker effect on hsp70 inhibition suggests that the effect is reversible and not due to a toxic effect on the cells.

Inhibition of hsp synthesis induced by sodium arsenite or Azc. The synthesis of hsp70 induced by exposing HeLa or COLO320 DM cells to sodium arsenite was also inhibited by quercetin treatment for 6 h before sodium arsenite was added (data not shown). When treated with Azc for 12 h, the synthesis of hsp110, hsp90, hsp70, hsp47 and grp78 was induced in HeLa cells (Fig. 4, lane 2). Again, the induction of these hsps was inhibited by the presence of 50 μM quercetin in the medium simultaneously (Fig. 4, lane 3). Although the response of COLO320 DM cells to Azc was weak, the induction of hsps was still inhibited by quercetin treatment (data not shown).

Inhibition of the induction of hsps by other flavonoids. The structures of quercetin and other flavonoids examined in this experiment are shown in Figure 5-A. Flavone and luteolin are flavones, quercetin and kaempferol are flavonols, and genistein is an isoflavone. Rutin is a 3-rhamno-glucoside of quercetin. The concentrations of these flavonoids added to the medium of COLO320 DM cells were selected from the point that the cell number did not increase during 3 days incubations, with the exception of rutin, which showed only about 20% of growth inhibition compared with the control cells up to a concentration of 300 μM. The final concentrations applied were 150 μM of flavone, 100 μM of genistein, 100 μM of kaempferol, 25 μM of luteolin and 300 μM of rutin.

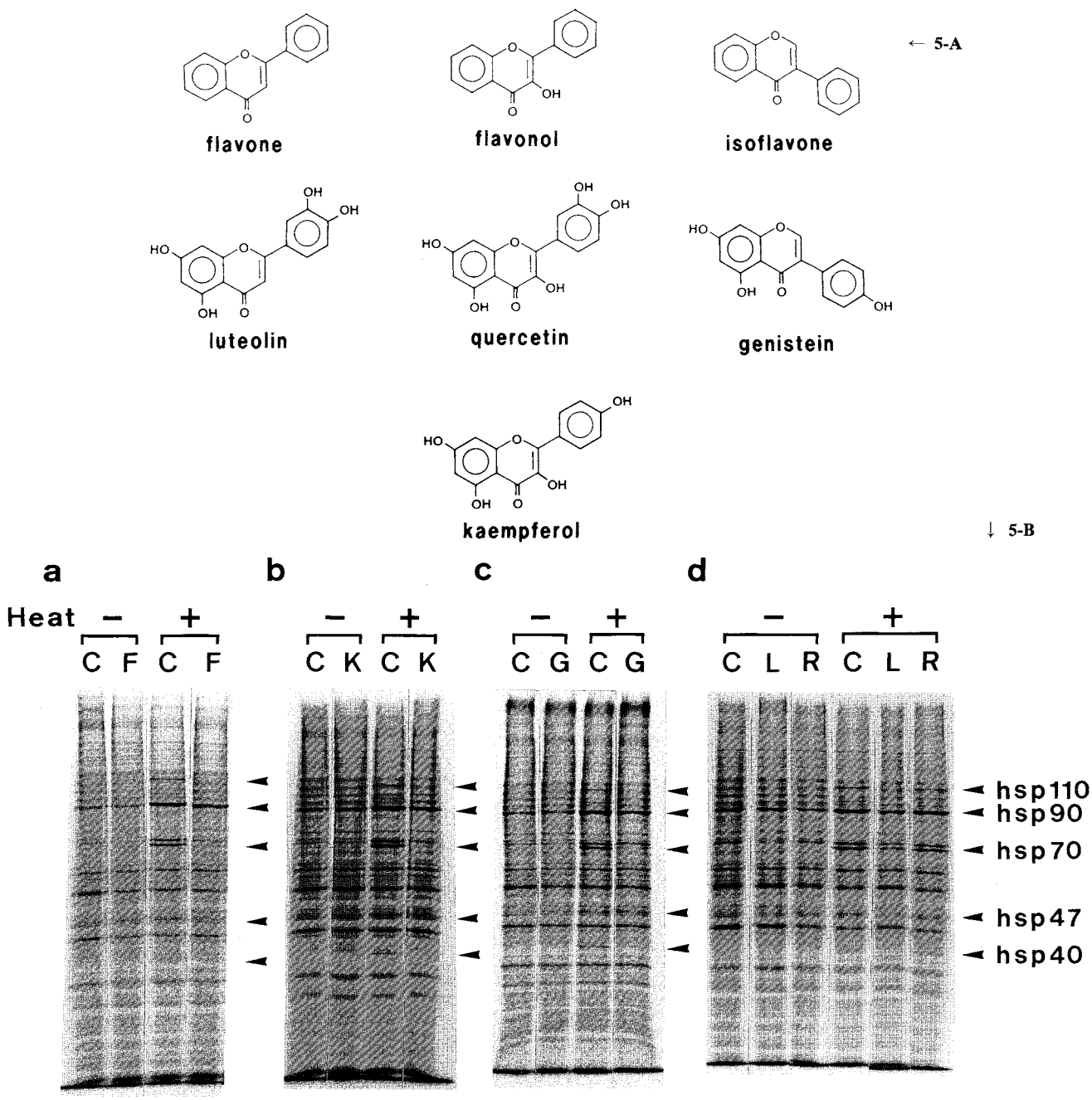

Fig. 5. Inhibition by other flavonoids of hsp induction. The chemical structures of flavonoids examined are shown in A. Flavone and luteolin are classified as flavones, quercetin, rutin (quercetin-3-rutinoside (=rhamnoglucoside)) and kaempferol are flavonols, and genistein is an isoflavonoid. The inhibitory effect of these flavonoids on hsp induction of COLO320 DM cells was studied by SDS-PAGE. B-a, 150 μM flavone (F); B-b, 100 μM kaempferol (K); B-c, 100 μM genistein (G); B-d, 25 μM luteolin (L) and 300 μM rutin (R). Arrowheads indicate the inducible hsps. Flavone, kaempferol and genistein inhibited the induction of hsp70, hsp110 and hsp40 almost to the same level as that without heat treatment, while luteolin inhibited the induction of hsp70 only slightly, and rutin hardly affected the induction of hsps.

The electrophoretic patterns of cell lysates treated with these flavonoids was almost the same as that of control cells (Fig. 5-B). When cells were heat-shocked, the induction of hsp110, hsp90, hsp70, p72, hsp47 and hsp40 was easily detected on SDS-PAGE slab gels. In the presence of flavone, kaempferol or genistein, the induction of hsp70, hsp110 and hsp40 was inhibited nearly to the level of that without heat shock. As for the

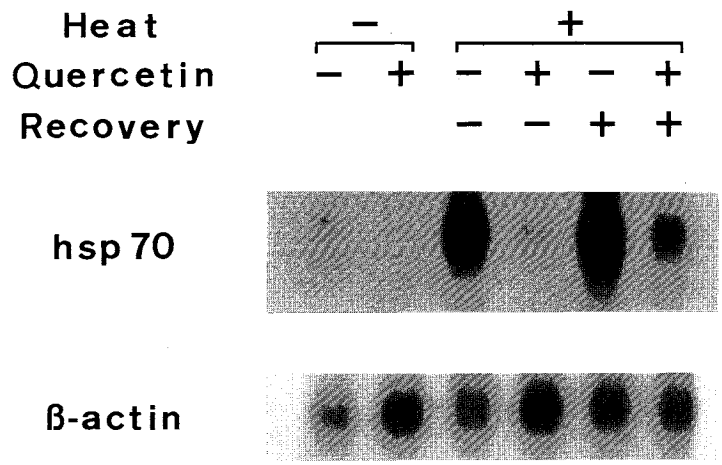

Fig. 6. Northern blot analysis of hsp70 mRNA. Cells were treated with or without heat shock in the presence or absence of quercetin. In the case of heat shock, total RNA was extracted immediately afterwards, or after the 2 h recovery period.

induction of hsp90 synthesis, kaempferol inhibited it as strongly as quercetin, while flavone and genistein inhibited it slightly weaker (Fig. 5-B-a, b, c). Luteolin caused a weak inhibition of hsp70 induction, and a moderate inhibition of other hsps (Fig. 5-B-d). Rutin hardly influenced the induction of all these hsps (Fig. 5-B-d). The synthesis of other proteins was virtually unaffected by these flavonoids.

Northern blot analysis. By Northern blot analysis, the effects of quercetin on the levels of hsp70 mRNA of COLO320 DM cells were examined by parallel analysis of β-actin mRNA as an internal control (Fig. 6). The level of hsp70 mRNA was markedly reduced by quercetin both immediately after heat-shock and after a 2 h recovery period. The low level of hsp70 mRNA at normal temperature was also reduced to an undetectable level, while β-actin mRNA remained virtually unchanged under all the conditions examined.

DISCUSSION

Although various stresses are known to induce the synthesis of hsps, such as heat, heavy metals, arsenite, hypoxia, ethanol, and amino acid analogues (19), a specific inhibitor of hsp synthesis has not been reported. Deuterium oxide and glycerol were reported to inhibit the synthesis of some hsps (8). However, inhibition by these reagents was thought to be due to the indirect effects caused by protecting thermolabile proteins of the cells from denaturation. Our results are thus the first report of a specific inhibition of hsp synthesis, the regulation of which is at the level of mRNA accumulation.

Quercetin has various biological activities as described in the introduction. Kaempferol is a flavonol, which is also widely distributed in plants, and has similar biological effect to quercetin. Genistein is known to be a specific inhibitor of tyrosine-specific protein kinases, while flavone has low activity (1). Quercetin and kaempferol also inhibit tyrosine kinases as strongly as genistein, while quercetin inhibits other kinase activities at the same time (9, 11). Luteolin has a stronger effect on growth inhibition of cultured cells than that of quercetin (22), and many of the biological activities of flavonoids are weakened in glycosilated compounds. Although structure-activity relationships have been reported in various biochemical and pharmacological activities of flavonoids, the inhibitory activity of hsp induction by several flavonoids shows no deducible correlation, and it seems difficult to relate this activity to a known mechanism.

Quercetin has been reported to act not only on cell membranes but also on a specific nuclear site, which is seen in the case of its interaction with estrogen type II binding sites (21). For the induction of hsps, conserved sequence known as heat shock elements (HSEs) in the promotor region and transacting heat shock factors (HSFs) are involved (19, 32, 39). Although the mechanism of inhibition of hsp synthesis by quercetin remains to be clarified, the possibility of the interaction of quercetin with HSEs or HSFs within the nucleus has been indicated by our preliminary experiments. The chloramphenicol acetyl transferase (CAT) gene ligated to the downstream of the promoter of hsp70 was transfected into COS cells. When the COS cells were heated in the presence of quercetin, CAT activity decreased compared with that in the absence of quercetin (Hosokawa, N., *et al.* manuscript in preparation).

Quercetin is reported to be a hyperthermic sensitizer for HeLa cells (12). Kim *et al.* discussed that this sensitization is due to a decrease in intracellular pH resulting from the inhibition of lactate transport. However, our results suggest another explanation: that the

inhibition of hsp(s) expression by quercetin causes the cells to be more sensitive to heat.

There are many reports which indicate that hsps are responsible for the acquisition of thermotolerance (17, 18), but which hsp(s) are responsible for thermotolerance remains to be determined. Since quercetin inhibits the induction of all the major hsps reported to date and has relatively low toxicity *in vivo* (2), it could also be utilized as a sensitizer in hyperthermic cancer therapy. In our preliminary experiments, quercetin inhibited the acquisition of thermotolerance in cells preheated at sublethal temperatures (Koishi, M., *et al.*, unpublished observation). Thus, quercetin and other flavonoids would be useful and worthwhile to study in terms of hyperthermia.

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