Regulation of heat shock protein synthesis by quercetin in human erythroleukaemia cells

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Synthesis of heat-shock proteins (HSPs) is universally induced in eukaryotic and prokaryotic cells by exposure to elevated temperatures or to other types of environmental stress. In mammalian cells, HSPs belonging to the 70 kDa family (HSP70) have a regulatory role in several cellular processes, and have been shown to be involved in the control of cell proliferation and differentiation. Although many types of HSP70 inducers have been identified, only a few compounds, all belonging to the flavonoid group, have been shown to inhibit HSP70 induction. Because inhibitors of HSP70 synthesis could be an important tool with which to study the function of this protein, we have investigated the effect of quercetin, a flavonoid with anti-proliferative activity which is widely distributed in nature, on HSP70 synthesis in human K562 erythroleukaemia cells after treatment with severe or mild heat shock and with other inducers. Quercetin was found to affect HSP70 synthesis at more than one level, depending on the conditions used. Indeed, after severe heat

shock (45 °C for 20 min) treatment with quercetin, at non-toxic concentrations, was found to inhibit HSP70 synthesis for a period of 3–4 h. This block appeared to be exerted at the post-transcriptional level and to be cell-mediated, as the addition of quercetin during translation of HSP70 mRNA *in vitro* had no effect. After prolonged (90 min) exposure at 43 °C, however, quercetin was found to inhibit also HSP70 mRNA transcription. Pretreatment of K562 cells with quercetin had no effect on HSP70 expression, and quercetin needed to be present during induction to be effective. Under all conditions tested, the quercetin-induced block of HSP70 synthesis was found to be transient and, after an initial delay, synthesis of HSP70 reached the control rate and continued at the same level for several hours after the time at which HSP70 synthesis had been turned off in control cells. Finally, inhibition of HSP70 synthesis by quercetin appeared to be dependent on the temperature used and on the type of stressor.

INTRODUCTION

The exposure of eukaryotic as well as prokaryotic cells to temperatures a few degrees above the physiological level triggers the synthesis of a particular set of proteins called heat-shock proteins (HSPs) (for a review, see Lindquist, 1986). The synthesis of HSPs represents an essential, finely regulated and highly conserved cellular response to adverse environmental conditions including, besides heat shock, the presence of amino acid analogues (Hightower, 1980), transition metal ions or sulphhydryl reagents (Levinson et al., 1980), glucose deprivation (Pouyssegur et al., 1977), hypoxia (Subjeck and Shyy, 1986) and virus infection [reviewed by Santoro et al. (1990)]. Synthesis of HSPs is also induced by biologically active molecules, including hemin (Singh and Yu, 1984), tumour necrosis factor α (König et al., 1992) and eicosanoids (Santoro et al., 1989; Köller and König, 1991).

HSPs can be divided into at least five families according to their molecular mass. The eukaryotic HSP70 genes encode a multi-gene family of proteins, including the major inducible HSP70, the constitutively expressed HSC70, the inducible HSP72, the glucose-responsive GRP78/BIP and the mitochondrial p75 [reviewed by Morimoto et al., (1992)]. Members of the HSP70 family are involved in the regulation of protein folding, assembly and intracellular translocation (constitutive HSP70), cyto-protection from the damaging effect of heat and refolding of partially denatured proteins (inducible HSP70), ATP-dependent catalysis of protein assembly/disassembly reactions (HSP70, groEL), disassembly of chlatrin-coated triskelia (HSC70) and binding to immunoglobulin heavy chains (GRP78) [reviewed by

Lindquist and Craig (1988)]. HSP70 proteins have also been shown to be involved in thermotolerance and in the control of cell proliferation and differentiation (Black and Subjeck, 1990; Santoro et al., 1990; Amici et al., 1993). Moreover, induction of HSP70 synthesis has been associated with the control of virus replication after treatment with heat shock (De Marco and Santoro, 1993) or with cyclopentenone prostaglandins (Amici and Santoro, 1991). However, no direct evidence for a causal relationship between HSPs and the control of cell proliferation or virus replication has been obtained, also because of the lack of specific inhibitors of HSP synthesis.

The discovery that the flavonoid quercetin, an inhibitor *in vitro* of 5-lipoxygenase and cyclo-oxygenase, is also an inhibitor of HSP synthesis in a human colon carcinoma cell line after heat shock (Hosokawa et al., 1990) and in human monocyte-macrophages during erythrophagocytosis (Kantengwa and Polla, 1991) could provide an important tool with which to analyse HSP70 functions.

In the present study we have investigated the effect of quercetin on HSP70 synthesis in human K562 erythroleukaemic cells after treatment with severe or mild heat shock and with other classical inducers. We report that, in the model studied, the effect of quercetin on HSP70 synthesis is transient, and is dependent on the temperature used and on the type of stressor.

EXPERIMENTAL

Materials

3,3',4',5,7-Pentahydroxyflavone (quercetin, Sigma Chemical Co., St. Louis, MO, U.S.A.) was dissolved in ethanol and stored as a

Abbreviations used: HSF, heat-shock factor; HSP, heat-shock protein; PGA₁, prostaglandin A₁.

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Figure 1 Dose-dependent inhibition of HSP70 synthesis by quercetin after severe heat shock

K562 cells, either kept at 37 °C (lane 1) or heat-shocked (45 °C, 20 min) in the absence (lane 2) or in the presence of vehicle (lane 3), or in the presence of various doses (lane 4, 20 μ M; lane 5, 30 μ M; lane 6, 40 μ M) of quercetin, were continuously labelled with L-[35 S]methionine for 6 h immediately after the heat-shock period. Cell lysates were processed for (a) SDS/PAGE analysis or (b) immunoblot analysis using a monoclonal anti HSP72/73 antibody. A quantitative evaluation of the radioactivity incorporated into the HSP70 band was carried out as described in the Experimental section.

1000-fold concentrated solution at -20 °C for a maximum of 1 week. Prostaglandin A₁ (PGA₁) (Cayman Chemical Co., Ann Arbor, MI, U.S.A.) was stored at -20 °C as a 10 mg/ml ethanolic stock solution. Sodium *meta*-arsenite (NaAsO₂), and L-azetidine 2-carboxylic acid were obtained from Sigma Chemical Co. HSP70 probe (pH 2.3 plasmid) was a kind gift of Dr. R. Morimoto (Northwestern University, Evanston, IL, U.S.A.).

Cell culture and treatment

Human erythroleukaemia K562 cells were grown in RPMI 1640 medium, supplemented with 10% (v/v) fetal calf serum and antibiotics at 37 °C in a 5% CO₂ humidified atmosphere.

For heating procedures, flasks were submersed in a temperature-controlled water bath (Grant Instruments, Cambridge, U.K.) at 45 $\pm$ 0.01 °C, 43 $\pm$ 0.01 °C or at 42 $\pm$ 0.01 °C for 20 or 90 min ($t_{1/2}$ = 1.5 min). Quercetin or the same volume of ethanol diluent were administered immediately before the beginning of heat shock or before addition to the media of the other stress-inducing agents, and maintained during treatment and the following recovery period, unless otherwise specified.

Protein synthesis and SDS/PAGE analysis

K562 cells subjected to heat shock in the presence or the absence of quercetin, as well as cells treated with other inducers, were subjected to L-[35 S]methionine continuous labelling or pulse labelling experiments, as described previously (Santoro et al., 1989). The radioactivity incorporated into trichloroacetic acid-insoluble material was determined and protein samples containing the same amount of radioactivity (unless otherwise specified) were separated by SDS/PAGE (10% w/v resolving gels) in a vertical slab gel apparatus, and processed for autoradiography as described (Santoro et al., 1989).

Densitometric analysis of the autoradiograms was performed on a BioRad model 620 CCD videodensitometer (BioRad Laboratories, Richmond, CA, U.S.A.) and the quantitative evaluation of proteins was determined using the BioRad 1-D Analyst software. HSP70 synthesis was expressed as percentage of total protein synthesis.

Quantitative Immunoblot analysis

Equal amounts of protein for each sample were separated by SDS/PAGE and blotted onto nitrocellulose. The filters were then incubated with a monoclonal anti-(72/73 kDa HSP) antibody (diluted 1:500) from HeLa cells (Amersham). The bound antibody was detected by horseradish peroxidase-linked sheep anti-mouse antibody, as previously described (Santoro et al., 1989). Selected bands were excised from the filters and the radioactivity was determined in a β -scintillation counter (LKB Instruments, Bromma, Sweden).

Northern blot analysis

Total cellular RNA was extracted following the procedure of Chomczynski and Sacchi (1987). After quantification of RNA by standard spectrophotometrical analysis, samples containing equal amounts of nucleic acid (15 μ g/lane) were fractionated on 1% (w/v) agarose-formaldehyde gels. The RNA was blotted from gels onto nylon membranes (Hybond N⁺, Amersham) and baked at 80 °C for 2 h. Membranes were prehybridized at 42.5 °C in 50% (v/v) formamide, 6 $\times$ SSC (1 $\times$ SSC = 15 mM NaCl, 0.15 M sodium citrate), 5 $\times$ Denhardt's solution, 0.1% (w/v) SDS, 100 μ g/ml denatured, fragmented salmon sperm DNA. Hybridization was performed at 42.5 °C in 10 ml of fresh prehybridization solution containing the HSP70 probe (pH 2.3 plasmid) (Wu et al., 1985), 32 P-labelled by a nick-translation procedure (Rigby et al., 1977). After stripping, filters were rehybridized with a 32 P-labelled probe derived from a cDNA fragment (1400 bp; *Pst*I) of the rat glyceraldehyde phosphate dehydrogenase gene as loading control. After hybridization, blots were washed twice in 6 $\times$ SSC for 15 min at room temperature, twice with 2 $\times$ SSC/0.2% (w/v) SDS at 65 °C for 20 min, and twice again with 0.2 $\times$ SSC/0.2% SDS at 65 °C for 20 min. Hybridized filters were exposed to Kodak X-AR5 films.

For measurement of total RNA synthesis, K562 cells subjected to heat shock in the presence or the absence of quercetin were labelled with [3 H]uridine (5 μ Ci/10⁶ cells) for 6 h after the end of the shock. Cells were washed, lysed and the radioactivity incorporated into TCA-insoluble material was determined.

In vitro translation

The *in vitro* translation assay employed 15 μ g of total cellular RNA. The reaction mixtures containing 24 μ l of nuclease-inactivated rabbit reticulocyte lysate (Amersham), L-[35 S]-Methionine (25 μ Ci/3 μ l; specific radioactivity > 1000 Ci/

mmol) and the RNA to be tested ($5 \mu\text{g}/\mu\text{l}$, $3 \mu\text{l}$) were incubated for 1 h at 30°C . Reaction was blocked by adding $6 \mu\text{l}$ of a solution containing $100 \mu\text{g}/\text{ml}$ pancreatic RNAase in 50 mM EDTA, pH 8.0. Samples were incubated for an additional 15 min at 37°C ; the same volume of 2-fold concentrated reducing PAGE sample buffer was then added, and samples were boiled for 5 min. Samples were analysed by SDS/PAGE and autoradiography as described above.

RESULTS

Effect of quercetin on HSP synthesis induction in heat-shocked K562 cells

To investigate the effect of quercetin on cell viability, protein synthesis and HSP70 synthesis, K562 cells (10^6 cells/ml) were

Table 1 Effect of quercetin pretreatment on HSP70 synthesis

K562 cells, pretreated with $30 \mu\text{M}$ quercetin or vehicle for 6 h at 37°C , were either kept at 37°C or subjected to heat shock (45°C , 20 min) in the presence of quercetin (+Q) or after the removal (—Q) of the drug, and labelled with L - $[^{35}\text{S}]$ methionine for 6 h. In treated samples, quercetin ($30 \mu\text{M}$) was present during labelling. Cell lysates were processed for SDS/PAGE and densitometric analysis. Data represent HSP70 synthesis expressed as percentage of total protein synthesis from a representative experiment. Three experiments were carried out with the same results.

Pretreatment	37°C		45°C	
	—Q	+Q	—Q	+Q
None	0.64	0.51	3.19	1.92
Quercetin	0.61	0.58	3.27	1.67

Figure 2 The quercetin-induced inhibition of HSP70 synthesis is temperature-dependent

K562 cells, either kept at 37°C or heat-shocked at 42°C (20 min) or at 45°C (20 min), were labelled continuously with L - $[^{35}\text{S}]$ methionine for 6 h soon after heat shock. (a) SDS/PAGE analysis shows that quercetin does not affect the protein pattern of cells kept at 37°C and does not inhibit HSP70 synthesis in 42°C -heat shocked cells. Lane 1, untreated; lane 2, 0.1% (v/v) ethanol; lane 3, $20 \mu\text{M}$ quercetin; lane 4, $30 \mu\text{M}$ quercetin; lane 5, $40 \mu\text{M}$ quercetin; C, untreated, not heat-shocked control cells. HSP70 is indicated by arrow. (b) Quantitative evaluation of HSP70 (○) and actin (●) synthesis by densitometric analysis of the samples in (a). (c) In a separate experiment, HSP70 synthesis was evaluated by quantitative immunoblot analysis as described in the Experimental section. □, untreated; ▨, 0.1% (v/v) ethanol; ▤, $20 \mu\text{M}$ quercetin; ▥, $30 \mu\text{M}$ quercetin; ▧, $40 \mu\text{M}$ quercetin. (c, inset) Protein synthesis in quercetin-treated cells was determined as radioactivity incorporated into TCA-insoluble material and expressed as percentage of control, untreated cells. ●, 37°C ; □, 42°C ; ○, 45°C .

Figure 3 Effect of quercetin on HSP70 synthesis in K562 cells after treatment with other stressors

(a) Replicate K562 cultures were treated (+) or not (–) with 30 μ M quercetin and soon after received the following treatment: lanes 1, control, 37 °C; lanes 2, 6 μ g/ml PGA_1 ; lanes 3, 2×10^{-5} M NaAsO_2 ; lanes 4; 2×10^{-3} M L-azetidine 2-carboxylic acid; lanes 5, heat shock, 42 °C for 20 min; lanes 6, heat shock, 45 °C for 20 min. Cells were labelled with L-[^{35}S]methionine soon after heat shock or soon after treatment with other stressors for the following 6 h, and then were processed for SDS/PAGE analysis. HSP70 is indicated by arrow. (b) Quantitative evaluation of radioactivity incorporated into the HSP70 protein after immunoblot analysis; bars 1–6 correspond with lanes 1–6 in (a). □, in the absence of quercetin; ▨, in the presence of quercetin (30 μ M).

treated with various doses of quercetin or ethanol diluent and immediately subjected to heat shock at 45 °C for 20 min. Cells were then resuspended in methionine-free RPMI 1640, supplemented with 2% (v/v) dialysed fetal calf serum, at the concentration of 10^6 cells/ml; quercetin was re-added where necessary, and cultures were incubated for 15 min at 37 °C. Control or quercetin-treated unstressed cells were treated identically. L-[^{35}S]Methionine was then added (final concentration 5 μ Ci/ml; specific radioactivity > 1000 Ci/mmol) and cultures were incubated at 37 °C for 6 h, after which time cells were collected. After determination of the radioactivity incorporated into trichloroacetic acid-insoluble material, samples were processed for SDS/PAGE analysis. Under these conditions, heat-shocked K562 cells synthesize a 70 kDa HSP (HSP70) and, to a lesser extent, HSPs belonging to the 90 and 110 kDa families (HSP90 and HSP110, respectively). Ethanol diluent had no effect on cell survival or HSP70 synthesis. Quercetin was found to inhibit HSP70 synthesis in a dose-dependent manner. HSP70 induction was completely blocked by quercetin at the concentrations of 50 and 100 μ M (results not shown). At these concentrations, however, quercetin treatment caused a dramatic inhibition of total protein synthesis after 6 h, as measured by L-[^{35}S]methionine incorporation into trichloroacetic acid-insoluble material (quercetin 50 μ M = 9%; 100 μ M = 3% of control), as well as of RNA synthesis, as measured by [^3H]uridine incorporation (quercetin 100 μ M = 13.6% of control), and eventually resulted in cell death (cell survival 24 h after heat shock: 50 μ M = 44.3%; 100 μ M = 42.7% of control). Doses of quercetin ranging from 20 to 40 μ M were instead effective in specifically inhibiting HSP70 synthesis (Figure 1) without affect-

ing cell viability, which was equal to control (> 98%, 24 h after treatment). The dose-dependent inhibition of HSP70 synthesis could also be detected by Western immunoblotting, followed by quantitative evaluation of HSP70 synthesis (Figure 1b). As previously reported in other cell lines (Hosokawa et al., 1990), pretreatment of K562 cultures with quercetin (30 μ M) up to 6 h before heat shock was not effective in blocking HSP70 synthesis, unless the drug was present in the medium during and after heat treatment (Table 1).

During normal growth conditions, quercetin (at the concentration of 20 μ M or higher) partially suppressed total protein synthesis (Figure 2c, inset) and potently inhibited K562 cell proliferation (Elia and Santoro, 1993). However, removal of quercetin (20 or 30 μ M) from the culture medium 24 h after treatment resulted in the return of protein synthesis to the control level and in a complete reversal of the cell proliferation block (Elia and Santoro, 1993). A similar inhibition of total protein synthesis was observed after 42 or 45 °C heat shock (Figure 2c, inset). SDS/PAGE analysis of samples containing equal amounts of radioactivity showed that at 37 °C quercetin had no effect on HSP70 synthesis and did not alter the overall pattern of proteins synthesized in K562 cells (Figures 2a and 2b). In K562 cells stressed at 45 °C, quercetin selectively and dose-dependently inhibited HSP70 synthesis, whereas, at all concentrations tested, it had no effect on HSP70 synthesis after a mild heat shock (42 °C, 20 min), suggesting differential mechanisms of induction (Figures 2a and b). These results were confirmed by quantitative evaluation of HSP70 synthesis after immunoblot analysis in cells kept at 37 °C or heat-shocked at 42 or 45 °C, in the presence of various doses of the drug (Figure 2c).

Figure 4 Quercetin-induced block of HSP70 synthesis is exerted at a post-transcriptional level

(a, b) Quercetin-treated (30 μM; lanes 2 and 4) or -untreated (lanes 1 and 3) K562 cells, either kept at 37 °C (lanes 1 and 2) or heat-shocked at 45 °C for 20 min (lanes 3 and 4) were pulse-labelled with L-[³⁵S]methionine for 30 min, starting 60 min after the end of heat shock. Cell lysates were subjected to SDS/PAGE (a) as described, and the autoradiographic patterns were subjected to densitometric analysis (b). (c, d) In the same experiment, total RNA was extracted from parallel cultures 90 min after the end of heat shock and employed in an *in vitro* translation assay: (c) SDS/PAGE analysis of *in vitro* translated proteins; (d) densitometric analysis of the autoradiographic patterns shown in (c). (e) K562 total cellular RNA was extracted before heat shock (lanes 1 and 2), soon after heat shock (3 and 4) or at 30 min (5 and 6), 60 min (7 and 8) and 90 min (9 and 10) after hyperthermic treatment in the presence (2, 4, 6, 8 and 10) or the absence (1, 3, 5, 7 and 9) of 30 μM quercetin. Samples were processed for Northern blot analysis using the ³²P-labelled pH 2.3 plasmid, for identification of HSP70 mRNA. Ethidium bromide staining of loaded RNA (15 μg/lane) is shown in the lower part of the figure. Northern blot analysis from a representative experiment is shown. Three experiments were carried out, with the same results. (f) Total RNA was extracted from K562 cells 90 min after heat shock and employed in an *in vitro* translation assay in the presence of 30 μM (lane 2) or 150 μM (lane 3) quercetin, or in the presence of ethanol vehicle (lane 1) as described in the Experimental section. No difference in the translation of HSP70 (indicated by arrow) was found between quercetin-treated and -untreated cells, even at the higher concentrations of quercetin.

Effect of quercetin on HSP70 synthesis after treatment with other inducers

Many substances are able to induce HSP synthesis in cultured cells. The ability of quercetin to interfere with HSP expression was tested in K562 cells treated with PGA₁, sodium *meta*-arsenite or L-azetidine 2-carboxylic acid. Soon after addition of quercetin (30 μM) or ethanol diluent to culture medium, K562 cells were treated with PGA₁ (6 μg/ml), NaAsO₂ (2 × 10⁻⁵ M) or L-azetidine 2-carboxylic acid (2.5 × 10⁻³ M). Parallel cultures were heat-shocked at 42 or 45 °C for 20 min, either in the presence or the absence of quercetin (30 μM); all cultures were then labelled with L-[³⁵S]methionine for 6 h and processed for autoradiography

or quantitative immunoblot analysis, as described above. Quercetin treatment caused a substantial reduction of HSP70 synthesis after exposure to PGA₁ (Figure 3, lanes 2), NaAsO₂ (Figure 3, lanes 3) or 45 °C heat shock (Figure 3, lanes 6). Quercetin treatment appeared not to affect the moderate induction of HSP70 synthesis after L-azetidine treatment (Figure 3, lanes 4) as well as after mild heat shock (Figure 3, lanes 5) in K562 cells.

Inhibition of HSP70 synthesis after short hyperthermic treatment at 45 °C by quercetin is exerted at a post-transcriptional level

To investigate whether the block of HSP70 synthesis by quercetin was exerted at a transcriptional or post-transcriptional

Figure 5 Effect of quercetin on the kinetics of HSP70 synthesis in K562 cells after severe heat shock

K562 cells were heat-shocked at 45 °C for 20 min in the absence (—Q) or in the presence (+Q) of 30 μ M quercetin. At the indicated times (1, 3, 5, 7 and 10 h after heat shock), aliquots of cells were collected from each culture and pulse-labelled as described in the Results section. (a) SDS/PAGE analysis of L-[³⁵S]methionine-labelled samples: C, control, not heat-shocked cells; HSP70 is indicated by arrow. (b) HSP70 synthesis in untreated (○) or quercetin-treated (●) heat-shocked cells was determined by densitometric analysis, and expressed as percentage of total protein synthesis.

level, two parallel sets of cultures were treated with quercetin (30 μ M) or ethanol diluent, and either kept at 37 °C or subjected to heat shock at 45 °C for 20 min and allowed to recover at 37 °C. An aliquot of the cells was employed to determine HSP70 synthesis by L-[³⁵S]methionine pulse labelling (20 μ Ci/sample, 30 min pulse) 1 h after the end of hyperthermic treatment, and the others were processed for RNA extraction at different times after heat shock. Total cellular RNAs were processed for Northern blot analysis and hybridized to radiolabelled HSP70 probe (pH 2.3 plasmid). As shown in Figure 4, whereas at 90 min from the beginning of the recovery period HSP70 protein synthesis was strongly inhibited in quercetin-treated cultures compared with untreated cells (Figures 4a and 4b, lanes 3 and 4), HSP70 mRNAs were equally expressed in quercetin-treated or -untreated heat-shocked K562 cells, either soon after hyperthermic treatment or at 30, 60 and 90 min after heat shock (Figure 4e).

Messenger RNAs from the same experiment, collected 90 min after heat shock, were translated *in vitro* to determine whether the HSP70 mRNA, transcribed in the presence of quercetin, could be altered so that its ability to be translated could be impaired. The results shown in Figure 4(c) and 4(d) demonstrate

that HSP70 protein was equally synthesized *in vitro* from mRNA derived from both quercetin-treated or -untreated heat-shocked K562 cells, suggesting a cell-mediated translational block. Moreover, addition of quercetin (30–150 μ M) during *in vitro* translation of mRNA extracted from K562 cells 90 min after a 20 min heat shock at 45 °C had no effect on HSP70 mRNA translation, indicating that the inhibition of HSP70 synthesis obtained *in vivo* is not a direct effect of quercetin but is cell-mediated (Figure 4f).

As described in the next section, after induction with prolonged (90 min) hyperthermic treatment at 43 °C, quercetin was found to inhibit HSP70 synthesis also at the transcriptional level.

Biphasic effect of quercetin on the kinetics of HSP70 synthesis

In order to investigate the effect of quercetin on the kinetics of HSP70 synthesis, K562 cells were subjected to either a 45 °C or a 42 °C heat shock (20 min) in the presence or the absence of 30 μ M quercetin. After heat shock, cultures were transferred to 37 °C and, at indicated times, 2 ml portions of cell suspensions were collected, pelleted and resuspended in the same volume of methionine-free RPMI 1640, containing 2 % (v/v) dialysed fetal calf serum and quercetin or control diluent. After 15 min of starvation at 37 °C, L-[³⁵S]methionine was added (10 μ Ci/ml) and cultures were incubated at 37 °C for 1 h. After labelling, cultures were processed for SDS/PAGE and autoradiography as described above. A 20 min treatment at 45 °C caused an induction of HSP70 synthesis, which was maximal between 2 and 5 h after heat shock, and went back to control levels by 10 h after treatment (Figures 5a and 5b). In quercetin-treated cells, however, synthesis of HSP70 reached the control rate and continued at the same level for several hours after HSP70 synthesis had been turned off in control cells. A second addition of 30 μ M quercetin 3 h after the first treatment was not effective in preventing HSP70 synthesis (results not shown).

The kinetics of HSP70 synthesis after a mild heat treatment (42 °C, for 20 min) was found to differ considerably from the results described above. HSP70 protein was found to be synthesized earlier, with a peak of maximal synthesis at 3 h after heat treatment, and to go back to the control level after 5 h. Again, quercetin treatment had no effect on the kinetics of HSP70 synthesis under these conditions (results not shown).

It has been previously reported that in human colon carcinoma cells, subjected to a 43 °C heat shock for 90 min, quercetin (100 μ M) blocks HSP70 transcription through inhibition of heat-shock factor activation. In order to investigate whether under these conditions HSP70 transcription was inhibited in erythroleukaemic cells, and, in this case, whether inhibition of HSP70 synthesis was transient or irreversible, K562 cells were subjected to hyperthermic treatment at 43 °C for 90 min in the presence or the absence of quercetin (100 μ M). Cells were labelled with L-[³⁵S]methionine (5 μ Ci, 1 h pulse) 2 h after treatment, and samples containing an equal amount of radioactivity were separated by SDS/PAGE and processed for autoradiography. In the same experiment, total cellular RNAs were extracted at 2 h after treatment and processed for Northern blot analysis. Under these conditions, quercetin was found to strongly inhibit both HSP70 synthesis and HSP70 mRNA transcription (Figures 6a and 6b). To study the effect of quercetin on the kinetics of HSP70 synthesis under these conditions, K562 cells treated with a 90 min, 43 °C heat shock in the presence or the absence of 100 μ M quercetin were pulse-labelled with L-[³⁵S]methionine (5 μ Ci, 1 h pulse) up to 12 h after treatment. Figure 6(c) shows that, also under these conditions, the quercetin-induced block

Figure 6 Effect of quercetin on HSP70 synthesis after prolonged (90 min) exposure at 43 °C

K562 cells were subjected to a prolonged (90 min) heat shock at 43 °C in the presence (+) or in the absence (–) of 100 μ M quercetin. After a 2 h recovery period at 37 °C, the cells were pulse-labelled with L-[35 S]methionine (5 μ Ci/ 10^6 cells) for 1 h and processed for autoradiography (a). In the same experiment, total RNA was extracted from parallel cultures 3 h after the beginning of heat shock and processed for Northern blot analysis, as described in the Experimental section. Ethidium bromide staining of loaded RNA (15 μ g/lane) is shown in the lower part of (b). (c) K562 cells were treated with 100 μ M quercetin or vehicle and then heat-shocked as described above. Replicate samples were pulse-labelled with L-[35 S]methionine for 1 h (5 μ Ci/ 10^6 cells) at different times during the recovery period at 37 °C. The samples were analysed by SDS/PAGE.

was transient, and after an initial delay, HSP70 synthesis reached the control level and was actually prolonged up to 12 h after heat treatment.

DISCUSSION

Flavonoids are a class of naturally occurring phenolic compounds that are ubiquitously present in vascular plants, fruits and vegetables (Kühnau, 1976). They are considered semi-essential food components because they are the most common and most active antioxidant compounds in our food (Heimann and Reiff, 1953), and synergize the effect of vitamin C in guinea pig and human tissues [reviewed by Kühnau (1976)]. It has been estimated that the average daily intake of flavonoids is ~ 1 g from a normal mixed diet. Thus, although absorption and catabolism mechanisms reduce the amount of flavonoids reaching the blood stream, these compounds can be looked upon as potentially important nutrients. Quercetin, which is one of the most widely distributed flavonoids in nature, has been reported to possess a wide range of biological activities on mammalian cell systems. It has been proved to be an inhibitor of cell growth in culture (Kim et al., 1984; Suolinna et al., 1975; Elia and Santoro, 1993), to impair glycolysis (Suolinna et al., 1974), and to inhibit macromolecular synthesis (Graziani and Chayoth, 1979), as well as the activity of a broad panel of enzymes, including protein kinases (End et al., 1987), ATPases (Kuriki and Racker, 1976), 5-lipoxygenase and cyclo-oxygenase (Laughton et al., 1991). Furthermore, quercetin has been reported to be a hyperthermic sensitizer in HeLa cells (Kim et al., 1984).

As anticipated in the Introduction, it has been recently reported that quercetin is also an inhibitor of heat-shock protein synthesis in a human colon carcinoma cell line after heat shock at 43 °C for

90 min (Hosokawa et al., 1990) and in human monocyte-macrophages during erythrophagocytosis (Kantengwa and Polla, 1991). In the first study, the ability of quercetin to inhibit the biosynthesis of HSP70 was found to be exerted by blocking HSP70 gene transcription (Hosokawa et al., 1990). The transcriptional block was reported to arise from quercetin interfering with the formation of the complex between the *trans*-acting heat-shock factor (HSF) and a *cis*-acting heat-shock element *HSE* in the promoter region of the gene (Hosokawa et al., 1992). The medium- and long-term effects of quercetin on heat-shock protein synthesis were not studied in these models.

The results described in the present report show that the effect of quercetin on HSP70 synthesis varies depending on the temperature used and on the type of stressor. K562 cells appear to be much more sensitive to quercetin than the other cell types reported in the literature, and doses of 50 or 100 μ M, used to obtain inhibition of HSP70 synthesis in different cell lines, appear not to be compatible with cell survival and/or maintenance of protein synthesis after heat shock in K562 cells. Concentrations of quercetin between 20 and 40 μ M were instead effective in specifically inhibiting HSP70 synthesis after severe (45 °C, 20 min) heat shock, without affecting cell viability. Under these experimental conditions, even though HSP70 synthesis was drastically reduced, HSP70 gene transcripts accumulated at similar rates in heat-shocked K562 cells, both in the presence or the absence of quercetin. Furthermore, HSP70 mRNAs from quercetin-treated or -untreated, heat-shocked K562 cells, could be translated *in vitro*, yielding comparable amounts of HSP70 protein. Finally, addition of quercetin during *in vitro* translation of HSP70 mRNA had no effect on HSP70 synthesis. These data suggest that, under these conditions, inhibition of HSP70 synthesis in quercetin-treated K562 cells is that the result of a post-

transcriptional block, is a cell-mediated event and is not attributable to quercetin-induced alterations of HSP70 mRNA.

The mechanism by which quercetin can selectively prevent HSP70 mRNA translation is not known. HSP70 mRNA from higher eukaryotes, and in particular from human cells, presents an altered translational specificity compared with other cellular mRNAs; it possesses high affinity for ribosome binding and is preferentially translated at increased ionic concentrations in the cytoplasm (Muñoz et al., 1984) or in conditions in which the translation of normal cellular RNAs is inhibited (Theodorakis and Morimoto, 1987; Muñoz et al., 1984; de Benedetti and Baglioni, 1986). It could be hypothesized that quercetin, which inhibits several translational activities, including the Na^+/K^+ -ATPase pump (Kuriki and Racker, 1976), could temporarily create conditions not favourable to HSP70 mRNA translation (i.e. changes in ionic concentration).

After a 90 min heat shock at 43 °C, however, the inhibition of HSP70 synthesis by treatment with quercetin (100 μM) was found to occur at the transcriptional level, as previously reported under these conditions (Hosokawa et al., 1990). These data indicate that quercetin can affect HSP70 synthesis at more than one level depending on at least three different factors: temperature, length of stress and concentration.

When K562 cells were subjected to mild heat shock (42 °C for 20 min), no differences in HSP70 synthesis could be detected between quercetin-treated or -untreated cultures. In mammalian cells, it has been demonstrated that the heat-shock transcriptional response to intermediate or extreme heat-shock temperatures is quite different. In the first case, the response is dependent on protein synthesis, is reversible upon return to control temperatures, and HSF readily converts to its control non-DNA-binding state; in the second, the response is cycloheximide-insensitive and results in a prolonged heat-shock gene transcription and HSF DNA-binding activity (Amici et al., 1992; Baler et al., 1992; Morimoto et al., 1992). In human cells, it has also been shown that incubation at 42 °C induces HSP70 synthesis with little effect on protein synthesis, whereas exposure to temperatures higher than 43 °C causes polysome disaggregation and an increase in free ribosomes, concomitant with a reduction in overall cellular protein synthesis (Theodorakis and Morimoto, 1987). It could be argued that a situation in which cellular protein synthesis is temporarily impaired favours quercetin inhibitory activity.

Quercetin-induced block of HSP70 synthesis was found to be transient under all conditions tested. In fact, after a 20 min heat shock at 45 °C, the production of HSP70 appears to begin ~ 3 h later in quercetin-treated cells than in the untreated control; after this time, HSP70 synthesis reached the control level and continued at the same rate for a period of several hours longer than control. Re-addition of quercetin 3 h after the initial treatment in the attempt to prolong the inhibitory activity was ineffective, suggesting that the reactivation of HSP70 synthesis was not caused by a decrease in the concentration of quercetin via binding, degradation or cellular export of this molecule. These results indicate that HSP70 synthesis was only delayed but not reduced by quercetin. Quercetin treatment actually resulted in prolonged and increased total synthesis of HSP70 protein. Similar results were obtained after a prolonged (90 min) heat treatment at 43 °C. Because it has been suggested that quercetin could be used to prevent HSP-induced thermotolerance in human cells (i.e. in cancer patients treated with hyperthermia) (Hosokawa et al., 1992), these data indicate that the long-term effects of quercetin treatment on both HSP70 synthesis and cell growth and metabolism should be carefully analysed.

The results described also show that quercetin-induced in-

hibition of HSP synthesis is dependent on the type of stressor. Indeed, whereas quercetin inhibited HSP70 synthesis induction by PGA_1 and NaAsO_2 , it had no effect on the moderate induction of HSP70 synthesis after L-azetidine treatment, indicating a selective activity. As HSP synthesis is also induced in human monocytes by hydrogen peroxide (Polla et al., 1987) or during generation of reactive oxygen species by human phagocytes during erythrophagocytosis (Clerget and Polla, 1990), it has been suggested that oxygen free radicals may play a role in HSP induction. It could be hypothesized that, because of its well-known antioxidant activity (Afanas'ev et al., 1989), quercetin could inhibit HSP synthesis in conditions of cellular oxidative stress. This possibility is supported by the different effect of quercetin during NaAsO_2 -induced as compared to L-azetidine-induced HSP70 synthesis. The selective inhibitory action of quercetin could then be a useful tool in the study of the regulation of the cellular response to different types of stressors.

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