Modulation of Prostaglandin A1-induced Thermotolerance by Quercetin in Human Leukemic Cells: Role of Heat Shock Protein 70

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ABSTRACT

Prostaglandins of the A type (PGAs) function as signals for heat shock protein (hsp) synthesis in mammalian cells. In human K562 erythroleukemic cells, PGA1 induces the synthesis of a M, 70,000 hsp (hsp70) by cycloheximide-sensitive activation of heat shock transcription factor (HSF). Induction of hsp70 has been associated recently with the ability of PGA to protect K562 cells from thermal injury, establishing a thermotolerant state; however, the role of hsp70 in thermotolerance is still controversial. Because quercetin was shown to modulate hsp70 expression after heat shock in K562 cells, we have investigated the effect of this flavonoid on HSF activation, hsp70 synthesis, and thermotolerance in human K562 cells after induction with PGA1. Quercetin was found to inhibit hsp70 synthesis for a period of 3–6 h after PGA1 treatment. This transient block was exerted at the transcriptional level and was not due to the loss of HSF DNA-binding activity. After the initial delay, hsp70 synthesis reached the same rate as the PGA1-treated control, and it was actually prolonged in the presence of quercetin. In PGA1-treated cells, quercetin suppressed PGA1-induced thermotolerance completely if the heat shock was applied at a time (6 h) when hsp70 synthesis was inhibited, whereas it could not prevent the establishment of a thermotolerant state if the heat challenge was applied 24 h after treatment, when hsp70 synthesis was not affected. These results support strongly the hypothesis that hsp70 is involved in the establishment of thermotolerance in human cells.

INTRODUCTION

PGs are a class of naturally occurring, cyclic, 20-carbon fatty acids, synthesized almost universally in eukaryotic cells via the cyclooxygenase-catalyzed metabolic reaction cascade originating from arachidonic acid and other polyunsaturated fatty acid precursors derived from the phospholipid pool of the cell membrane. PGs play a regulatory role in a wide variety of physiopathological processes, including cell proliferation and differentiation (1), the immune response (2), inflammation (3), cytoprotection (4, 5), hyperthermia (6), and virus replication (7). In particular, PGs containing an ω-unsaturated carbonyl group in the cyclopentane ring of the molecule (cyPGs) have been reported to exert an inhibitory activity on the proliferation of tumor cells in different experimental models (reviewed in Refs. 8 and 9), by provoking a block along the cell cycle progression, in G1 (10). cyPGs also have been shown to possess a potent antiviral activity against a wide variety of DNA and RNA viruses (11) and to protect human cells from thermal injury, establishing a thermotolerant state, which lasts for 24–48 h (12).

Thermotolerance, the acquisition of a transient increased resistance to heat, is a universal noninheritable phenomenon, induced in cells generally by exposure to a nonlethal hyperthermic treatment (13). Human cells expressing thermotolerance can exhibit survival levels several hundred times higher than normal cells when exposed to lethal hyperthermic temperatures (14). The development of thermotolerance in tumor cells is one of the major concerns in the treatment of human cancer with hyperthermia (15), and, for this reason, the molecular mechanism responsible for the development of thermotolerance has been investigated actively.

Exposure to elevated temperatures, as well as to other stressing agents, rapidly triggers the onset of a defensive response characterized by the synthesis of a set of polypeptides, the hsps. The activation of heat shock gene transcription is mediated by the HSF, which binds to HSEs in the promoters of heat shock genes. Several HSFs have been identified in mammalian cells (16). Activation of HSF1, which is the primary component of the HSF DNA-binding activity present in cells exposed to heat shock, requires oligomerization, acquisition of DNA-binding activity, and localization to the nucleus (16). The human HSF1 also has been shown to be phosphorylated on heat stress (16, 17).

hsps can be divided into at least five families depending on their molecular weight. The eukaryotic hsp70 genes encode a multigene family of proteins, including the major inducible hsp70, the constitutively expressed hsc70, the inducible hsp72, the glucose-responsive grp78-BiP, and the mitochondrial p75 (16). Members of the hsp70 family have been involved in the regulation of protein folding, assembly and intracellular translocation, cytoprotection from the damaging effect of heat and refolding of partially denatured proteins, ATP-dependent catalysis of protein assembly-disassembly reactions, disassembly of chlatin-coated triskelia, and binding to immunoglobulin heavy chains (grp78-BiP; reviewed in Ref. 18). hsp70 proteins also have been involved in thermotolerance (19); however, no definitive evidence regarding a causal relationship between hsps and thermotolerance has been achieved to date, also due to the lack of specific inhibitors of hsp synthesis.

cyPGs have been shown to function as signals for hsp70 induction in a wide variety of mammalian cells (8, 20); induction of hsp70 gene transcription by PGAs is mediated by cycloheximide-sensitive activation of HSF (21). Activation of HSF1, but not HSF2, by PGA has been shown recently in K562 cells.4

To investigate the possible role of hsp70 in PGA-induced thermotolerance, we have studied the effect of the flavonoid quercetin, which was reported to inhibit hsp70 synthesis (22, 23) by interfering with the formation of the complex between the cis-acting HSEs in the promoter region of the hsp70 gene and HSF (24). Recently, we have shown that quercetin is able to modulate hsp70 expression in K562 erythroleukemic cells after heat shock (25). Quercetin was found to inhibit hsp70 synthesis transiently. However, after an initial delay, quercetin was found to prolong hsp70 synthesis in these cells, actually causing an increase in the intracellular levels of this protein. In the present report, we show that, also in the case of hsp70 induction by PGA1, quercetin inhibits hsp70 transcription and translation in K562 cells.

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6 The abbreviations used are: PG, prostaglandin; hsp, heat shock protein; HSF, heat shock transcription factor; HSE, heat shock element; cyPG, cyclopentenone PG; CSA, colony survival assay; GAPDH, glyceraldehyde phosphate dehydrogenase; MDP, Molecular Dynamics Phospholmage; GMSA, gel mobility shift assay; RT, room temperature.

4 A. Rossi and M. G. Santoro, unpublished observation.
cells for a period of several hours, after which time hsp70 is synthesized at the same rate of control. In PGA1-treated cells, quercetin inhibited PGA1-induced thermotolerance dramatically if the challenge heat shock was applied at a time (6 h) when hsp70 synthesis was still inhibited by the flavonoid, whereas it could not prevent thermotolerance if the heat challenge was applied 24 h after treatment, when hsp70 synthesis was not affected by quercetin.

MATERIALS AND METHODS

Cell Culture and CSA. Human erythroleukemia K562 cells were grown in RPMI 1640 medium, supplemented with 10% FCS and antibiotics at 37°C in a 5% CO2 humidified atmosphere. 3,3',4',5,7-Pentahydroxyflavone (quercetin; Sigma Chemical Co., St. Louis, MO) was dissolved in ethanol and kept at -20°C for a maximum of 1 week. PGA1 (Cayman Chemical Co., Ann Arbor, MI) was stored at -20°C in absolute ethanol. For CSA, at different times after PGA1 addition, cells were exposed to a hyperthermic treatment at 45°C for a variable length of time.

For the heating procedure, flasks were submersed in a temperature-controlled water bath (Grant Instruments, Cambridge, United Kingdom) at 45 ± 0.01°C. Soon after heat shock, cells were plated in 35-mm Petri dishes at the density of 1 × 10^6 cells/dish in culture medium containing 30% FCS and 0.2% Bacto Agar and incubated at 37°C for 13–16 days. Only colonies of 100 or more cells were counted. Every sample was tested in duplicate, and each experiment was repeated at least three times. Survival values were adjusted for the plating efficiency of the appropriate control. Plating efficiencies were 60% for control and PGA1-treated cells and 45% for quercetin-treated counterparts.

Statistical analyses were performed using a nonparametric test for unpaired data. Data are expressed as means ± SD, and P < 0.05 was considered significant.

Protein Synthesis and PAGE Analysis. K562 cells treated with PGA1, in the presence or absence of quercetin were labeled with [35S]methionine (5 μCi/10^6 cells) as described previously (20). After cell lysis, the radioactivity incorporated into trichloroacetic acid-insoluble material was determined, and protein samples containing the same amount of radioactivity were separated by SDS-PAGE in a vertical slab gel apparatus and processed for autoradiography as described (20). Densitometric analysis of the autoradiograms was performed on a Bio-Rad 620 CCD video densitometer (Bio-Rad Laboratories, Richmond, CA), and the quantitative evaluation of proteins was performed using the Bio-Rad 1-D Analyst software. β-Actin levels were determined as controls. hsp70 synthesis was expressed as a percentage of total protein synthesis.

Immunoblot Analysis. Equal amounts of protein for each sample were separated by SDS-PAGE and blotted to nitrocellulose. The filters were then incubated with a monoclonal anti-hsp70 antibody (Amersham; diluted 1:500) from HeLa cells or with a rabbit polyclonal antibody against the human HSF1 (kindly provided by Dr. R. Morimoto, Northwestern University, Evanston, IL). The bound antibody was detected by horseradish peroxidase-linked secondary antibodies, as described previously (20). For quantitative immunoblot analysis, selected bands were excised from the filters, and the radioactivity was determined in a β scintillation counter.

Northern Blot Analysis. Total cellular RNA was extracted following the procedure of Chomczynski and Sacchi (26). After quantification of RNA by standard spectrophotometric analysis, samples containing equal amounts of nucleic acid (15 μg/lane) were fractionated on 1% agarose-formaldehyde gels. The RNA was blotted from gels onto nylon membranes (Amersham Hybond N+) and baked at 80°C for 2 h. Membranes were prehybridized at 42.5°C in 50% formamide, 6× SSC (1× SSC = 15 mM NaCl and 0.15 M sodium citrate), 5× Denhardt’s solution, 0.1% SDS, and 100 μg/ml denatured, fragmented salmon sperm DNA. Hybridization was performed at 42.5°C in 10 ml fresh prehybridization solution, containing the 32P-labeled hsp70 probe, prepared by random-primed DNA synthesis of the human hsp70 gene (plasmid pH2.3; Ref. 27). After stripping, filters were rehybridized with a 32P-labeled probe derived from a cDNA fragment (1400 bp; PstI) of the rat GAPDH gene as a loading control. After hybridization, blots were washed twice in 6× SSC for 15 min at room temperature, twice with 2× SSC and 0.2% SDS at 65°C for 20 min, and twice again with 0.2× SSC and 0.2% SDS at 65°C for 20 min. Hybridized filters were exposed to Kodak X-AR5 films, and radioactivity was quantified by using a MDP analyzer.

GMSA. Whole-cell extracts were prepared from aliquots of 10^7 K562 cells, either untreated or treated with PGA1 (6 μg/ml) in the presence or the absence of 30 μM quercetin, collected at various times after drug addition, as described (21). Extracts (10 μg/sample) were mixed with 0.1 ng 32P-labeled HSE oligonucleotide and 0.5 μg poly(dIdC) (Pharmacia) in 25 μl binding buffer [10 mM Tris-Cl (pH 7.8), 30 mM NaCl, 1 mM EDTA, 0.5 mM DTT, and 5% glycerol]. After a 30-min incubation at RT, HSF-HSE complexes were analyzed by nondenaturing 4% PAGE and autoradiography as described (21), and the amount of the shifted HSE probe was quantified by MDP analysis. For in vitro HSF DNA-binding inhibition experiments, extracts from 10^7 K562 cells, either untreated or treated with PGA1 (6 μg/ml), were added to the binding reaction mixture pretreated for 15 min at RT with 30, 50, or 100 μM quercetin or the corresponding amount of ethanol diluent. After an additional 30-min incubation at RT, HSF-HSE complexes were detected as described above.

RESULTS

Effect of Quercetin on PGA1-induced hsp70 Synthesis and hsp70 mRNA Accumulation in K562 Cells. Quercetin has been shown recently to modulate hsp70 synthesis induction after heat shock or other types of stress in K562 cells (25). To investigate whether quercetin inhibits hsp70 synthesis after PGA1-induction, K562 cells...
were treated with various concentrations of quercetin (20, 30, and 40 μM) or the corresponding amount of ethanol, and immediately after quercetin treatment, PGA₁ (6 μg/ml) or an equal amount of control diluent was added to the culture media. Cells were then labeled with L-[35S]methionine for the next 6 h. The addition of quercetin to PGA₁-treated K562 cells did not alter the level of protein synthesis significantly compared with ethanol-treated controls. Samples containing the same amount of radioactivity were processed for SDS-PAGE and autoradiography. As expected, PGA₁ treatment induced hsp70 synthesis (Fig. 1). PGA₁ increased hsp90 levels only moderately (less than 50%) starting 12 h after treatment, and it did not alter hsp110 synthesis, as determined by densitometric analysis of the autoradiograms. To investigate whether PGA₁ was affecting the synthesis of the low-molecular-weight hsp27, which is normally labeled poorly with L-[35S]methionine (28), K562 cells treated as described above were labeled with L-[3H]leucine (12 μCi/10⁶ cells; 3-h pulses) at different times after the addition of PGA₁. As described previously in murine cells (29), PGA₁ did not induce hsp27 synthesis in K562 cells up to 9 h after the beginning of treatment (data not shown).

In the absence of PGA₁, quercetin did not alter the pattern of the proteins synthesized at all concentrations tested (25). At 6 h after induction, hsp70 synthesis was reduced significantly by quercetin in PGA₁-treated cells (Fig. 1A) even at the lower concentration tested (20 μM). The reduction in hsp70 synthesis was quantified after Western immunoblot analysis with anti-hsp70 monoclonal antibodies by evaluating the radioactivity incorporated into the hsp70 band, as described in "Materials and Methods." (Fig. 1, B and C.)

To investigate whether the block of hsp70 synthesis by quercetin was exerted at a transcriptional or post-transcriptional level, two sets of cultures were treated either with quercetin (30 μM) or ethanol diluent immediately prior to PGA₁ (6 μg/ml) addition. Aliquots of cells were collected and processed for RNA extraction at different times after PGA₁ addition. Total cellular RNAs were subjected to Northern blot analysis and hybridized to a radiolabeled hsp70 probe (pH2.3 plasmid). As shown in Fig. 2A, the level of hsp70 mRNA accumulation after PGA₁ induction was decreased substantially in quercetin-treated K562 cells compared with controls up to 180 min at 37°C. In the same conditions, GAPDH gene mRNA expression was not affected by the presence of the flavonoid. To determine whether the reduced hsp70 mRNA accumulation in quercetin-treated cells could be a consequence of an increased turnover of the hsp70 mRNA, two sets of cultures were treated with quercetin (30 μM) or ethanol diluent, immediately prior to PGA₁ (6 μg/ml) addition. The HS genes were allowed to transcribe for 2 h after PGA₁ addition. At this time, the transcription inhibitor actinomycin D (10 μg/ml) was added to the cultures, and the total cytoplasmic RNA was extracted at different times and processed for Northern blot analysis. Levels of hsp70 mRNA were much lower in quercetin-treated compared with untreated cells; however, as shown in Fig. 2B, quercetin treatment did not alter the rate of hsp70 mRNA degradation.

Effect of Quercetin on HSF Activation in PGA₁-treated K562 Cells. Induction of hsp70 gene transcription by PGA₁ in K562 cells has been shown to be mediated by cycloheximide-sensitive activation of the HSF (21). In a different cell system, quercetin has been shown to prevent the formation of the complex between HSF and the HSE in the promoter region of the hsp70 gene (24). Therefore, we have investigated the possibility that quercetin was actually interfering with the HSF-HSE complex formation in K562 cells after PGA₁ addition. As shown previously (21), HSF-HSE complex formation was detected between 1 and 2 h after PGA₁ treatment (Fig. 3, A and B), attained maximal levels between 5 and 7 h, and declined after 9 h (Fig. 4). As described previously for heat shock induction (17), HSF DNA-binding activity in PGA₁-treated cells was associated with a significant increase (approximately M, 7,000) in HSF molecular size, as shown by Western blot analysis using polyclonal anti-HSF1 antibodies (Fig. 3C). The increase in molecular weight of HSF1 after induction has been shown previously to be the result of HSF1 phosphorylation during activation (17).
Quercetin by itself was unable to trigger the activation of HSF (Fig. 3A). The presence of quercetin (30 μM) during PGA1-treatment (6 μg/ml) did not alter either the kinetics or the extent of the HSF binding to a synthetic 32P-HSE oligonucleotide in a gel mobility shift assay up to 9 h after PGA1 addition in K562 cells (Figs. 3, A and B, and 4). However, in the presence of quercetin, a decrease in the amount of the high-molecular-size form of HSF1 was detected (Fig. 3C), suggesting that treatment with the flavonoid causes a decrease in HSF1 phosphorylation. Moreover, the presence of quercetin seemed to maintain HSF in an activated DNA-binding state up to 24 h after PGA1 addition (Fig. 4).

The addition of quercetin (30–100 μM) during the in vitro binding assay had no effect on PGA1-induced HSF activation (Fig. 5), suggesting that quercetin is not interfering directly with the HSF-HSE complex formation.

Biphasic Effect of Quercetin on the Kinetics of hsp70 Synthesis in PGA1-treated K562 Cells. We have shown previously that treatment with quercetin cannot prevent but only delays hsp70 induction by heat shock in K562 cells. In fact, after a 2–3-h delay, hsp70 synthesis continues in the presence of quercetin for at least 3 h longer than in control cells (25). We then studied the effect of quercetin on the time course of hsp70 protein synthesis after PGA1 treatment in K562 cells. Interestingly, not only the extent but also the duration of hsp70 synthesis was found to depend on the concentration of the PG added to the medium. After treatment with PGA1 at the concentration of 6 μg/ml, hsp70 synthesis peaked at 8 h and was turned off by 12 h after PGA1 addition (Fig. 6). At the concentration of 10 μg/ml, PGA1-induced hsp70 synthesis began after 1 h and continued up to 24 h after the beginning of treatment (Fig. 7). The simultaneous presence of quercetin (30 μM) during PGA1 treatment was found to delay hsp70 synthesis and decrease hsp70 levels for a period of 6–9 h after the addition of PGA1, at the concentration of 6 or 10 μg/ml, respectively. After the initial delay, hsp70 synthesis was actually prolonged in the

![Figure 3](image-url)

**Fig. 3.** Effect of quercetin on PGA1-induced HSF activation. A, K562 cells, in the presence of quercetin (30 μM; +) or ethanol diluent (−), were treated with PGA1 (6 μg/ml; Lanes 3–10) or control diluent (Lanes 1 and 2). At different times after PGA1 addition (30 min, Lanes 3 and 7; 60 min, Lanes 4 and 8; 120 min, Lanes 5 and 9; 180 min, Lanes 6 and 10) or at 180 min after control diluent addition (Lanes 1 and 2), cells were collected and frozen quickly in liquid nitrogen. Whole-cell extracts were analyzed for HSF DNA-binding activity by GMSA, as described in “Materials and Methods.” HSF, induced form of DNA-binding activity; CHBA, constitutive HSE-binding activity; NS, nonspecific protein-DNA interactions. B, quantitative evaluation of HSF-HSE complex formation by MDP analysis in samples treated with 30 μM quercetin (●) or ethanol control (○). Values are expressed as percentage of maximum level. C, Equal amounts of protein (50 μg) from samples described in A were separated by SDS-PAGE (8% polyacrylamide gel) and blotted to nitrocellulose. Immunodetection of HSF1 was performed with a rabbit polyclonal antibody.

![Figure 4](image-url)

**Fig. 4.** Quercetin prolongs PGA1-induced HSF activation in K562 cells. A, K562 cells were treated with quercetin (30 μM; +) or ethanol diluent (−) immediately before PGA1 (6 μg/ml; Lanes 2–11) addition. At different times after PGA1 addition (0 h, Lanes 1; 3 h, Lanes 2 and 7; 5 h, Lanes 3 and 8; 7 h, Lanes 4 and 9; 9 h, Lanes 5 and 10; 24 h, Lanes 6 and 11), whole-cell extracts were analyzed for HSF DNA-binding activity by GMSA, as described in the legend to Fig. 3. B, quantitative evaluation of HSF-HSE complex formation by MDP analysis in samples treated with 30 μM quercetin (●) or ethanol control (○). Values are expressed as percentage of maximum level.
Quercetin modulates PGA1-induced thermotolerance

For heat resistance by CSA, as described in "Materials and Methods." At both 6 and 24 h, PGA1 was found to protect cells from thermal injury (Fig. 9). As reported previously (12), maximum protection was found 24 h after PGA1 treatment. Quercetin alone had no effect on thermotolerance in the absence of PGA1 treatment. In PGA1-treated cells, quercetin caused a total loss of PGA1-induced thermal protection if the lethal heat shock was applied at a time (6 h) when hsp70 synthesis was still inhibited by quercetin, whereas it affected cell thermotolerance only moderately if the challenge heat shock was applied 24 h after treatment, when hsp70 synthesis was not inhibited by the flavonoid (Fig. 9). Similar results were obtained in the case of heat-induced thermotolerance in the same cells (results not shown).

Discussion

Flavonoids, a class of naturally occurring phenolic compounds ubiquitously present in vascular plants, fruits, and vegetables (31), are considered semielemental food components, being the most common and most active antioxidant compounds in our food (32) and syner-

Fig. 5. Effect of quercetin on HSF-HSE complex formation in vitro. Whole-cell extracts from 10^5 K562 cells untreated (C) or treated with PGA1 (6 µg/ml) for 3 h (PGA1) were added to the GMSA binding reaction mixture, pretreated for 15 min at RT with 30 (Lanes 2 and 6), 50 (Lanes 3 and 7), or 100 (Lanes 4 and 8) µM quercetin or ethanol diluent (Lanes 1 and 5). HSF-HSE complexes were detected after 30 min incubation at RT.

Presence of quercetin. The length of the period in which hsp70 synthesis was prolonged also depended on the concentration of PGA1 used (Figs. 6 and 7).

To investigate whether the transient nature of the quercetin inhibitory effect could be due to metabolic inactivation of the drug, K562 cells were treated with PGA1 (6 µg/ml) in the presence or absence of 30 µM quercetin or control diluent. Quercetin treatment was repeated after 3 h in some of the cultures, and hsp70 synthesis was determined 7 h after the beginning of PGA1 treatment by labeling with L-[35S]methionine (5 µCi/10^6 cells; 1-h pulse). At this time, the levels of hsp70 synthesis were found to be similar to the control even in K562 cells, which had received a second treatment with quercetin (data not shown), indicating that the reversal of the inhibitory effect was not due to quercetin metabolic inactivation.

Effect of Quercetin on PGA1-induced Thermotolerance in K562 Cells. We examined the effect of quercetin on K562 cell proliferation and thermotolerance. Cells were seeded in RPMI 1640 culture medium (2 × 10^5 cells/ml) in the presence of different concentrations of quercetin or ethanol diluent. Cell numbers and viability were determined daily for a period of 96 h. Quercetin was found to suppress K562 cell proliferation completely at the concentration of 30 µM (Fig. 8, A and B). Whereas removal of the drug 24 h after the beginning of treatment resulted in a rapid return of the cell proliferation rate to control levels (Fig. 8B, inset), indicating a cytostatic rather than cytotoxic effect, treatments for longer periods resulted in the progressive loss of growth potential, which was irreversible after a 144 h incubation in the presence of the drug (30). At the concentration of 30 µM, quercetin inhibited protein synthesis only moderately for 24 h after treatment, as shown by L-[35S]methionine incorporation into trichloroacetic acid-insoluble material (control, 2.88 ± 0.05 × 10^5 cpm/10^5 cells; quercetin, 2.40 ± 0.07 × 10^5 cpm/10^5 cells).

For thermotolerance studies, replicate K562 cultures were treated with PGA1 or ethanol diluent in the presence or absence of 30 µM quercetin and incubated at 37°C. At 6 or 24 h after PGA1 addition, aliquots of the different cultures were subjected to a challenge heat shock at 45°C for 15, 30, 45, or 60 min, respectively, and cultures were tested...
gizing the effect of vitamin C in human tissues (reviewed in Ref. 31). Quercetin, which is one of the most widely distributed flavonoids in nature, has been reported to possess a wide range of biological activities in mammalian cells. In fact, it has been shown to inhibit cell growth in culture (30, 33, 34), to impair glycolysis (33), and to inhibit macromolecular synthesis, as well as the activity of several enzymes, including ATPases (35), 5-lipoxygenase and cyclo-oxygenase (36), and protein kinases (37). Quercetin also has been reported to be a hyperthermic sensitizer in HeLa cells (38).

Quercetin has been shown to inhibit hsp synthesis after heat shock in a human colon carcinoma cell line and HeLa cells (22), and in human monocyte-macrophages during erythrophagocytosis (23). In human tumor cells, inhibition was found to be exerted at the transcriptional level, and it was related to a dose-dependent inhibition of HSF activation (24). In the same cell lines, the inhibition of hsp70 induction was associated with a loss of ability to acquire a thermotolerant state induced by heat shock, suggesting that quercetin could improve the efficacy of clinical fractionated hyperthermia (39). We have reported recently that in human K562 cells, the effect of quercetin on hsp70 induction varies depending on the type of stressor and on the temperature used during heat shock (25). Moreover, quercetin could inhibit hsp70 synthesis after heat shock only temporarily, and, after an initial delay, the presence of the flavonoid was found to prolong hsp70 synthesis in K562 cells, actually causing an increase in the intracellular level of this protein (25).

Fig. 7. Effect of quercetin treatment on the kinetics of hsp70 synthesis in K562 cells treated with 10 μg/ml PGA1. A, K562 cells were treated with 10 μg/ml PGA1 in the presence (+ Q) or the absence (− Q) of quercetin (30 μM). At the indicated times, aliquots of cells were collected and labeled with L-[35S]methionine (5 μCi/10⁶ cells; 1-h pulse). Samples containing equal amounts of radioactivity were processed for SDS-PAGE. B, hsp70 synthesis in the presence (■) or absence (○) of quercetin was determined by densitometric analysis and expressed as percentage of total protein synthesis. Levels of β-actin were not altered at any time in the same experiment (data not shown).
Drug 24 h after the beginning of treatment resulted in the return of the phenotype in these cells (42). The same result could be achieved, also by the presence of the flavonoid, HSF remained in an activated, DNA-binding state longer than in the PGA1-treated control. This is in contrast with the results described previously by Hosokawa et al. (24), who reported that HSF DNA-binding activation was inhibited by quercetin in COLO 320DM cells, a cell line derived from a human colon cancer. This difference could be due either to a cell type-specific activity of quercetin, to the different type of inducer or to the different concentration of quercetin used, because inhibition of HSF DNA-binding activity by quercetin in COLO cells was detected at higher concentrations (50–100 μM), which were found to be toxic in K562 cells.

In K562 cells, quercetin appeared to inhibit HSF1 phosphorylation at early times after PGA1 induction. In fact, a nonselective inhibition of mammalian protein kinase activity by quercetin has been described (37). However, increases in phosphorylation have been reported not to be absolutely required for acquisition of HSF1 transcriptional activity (17), and whether other steps of the hsp70 gene transcriptional activation process, such as translocation to the nucleus, could be impaired by the presence of quercetin needs to be investigated further.

After the initial delay, hsp70 synthesis was found to be actually prolonged in the presence of quercetin in PGA1-treated K562 cells. At the concentration of 30 μM, quercetin inhibited protein synthesis only moderately, whereas, as reported previously in different cell lines (33, 34), it suppressed K562 cell proliferation completely. Removal of the drug 24 h after the beginning of treatment resulted in the return of the cell proliferation rate to the control levels, whereas longer treatments resulted in the progressive loss of growth potential. At this concentration, quercetin did not affect the thermosensitivity of K562 cells. In PGA1-treated cells, quercetin suppressed thermotolerance completely when the challenge heat shock was applied at a time (6 h) at which hsp70 synthesis was inhibited effectively by the flavonoid. This effect does not seem to be related either to quercetin antiproliferative activity or to a cytotoxic effect, because removal of the flavonoid up to 24 h after the beginning of treatment resulted in restoring the K562 cell growth rate, and protein synthesis was not suppressed significantly up to 24 h after quercetin addition. A lack of cytotoxicity also is indicated by the fact that exposure to quercetin for longer periods (24 h) of time did not result in the total loss of thermotolerance. In fact, when the lethal heat challenge was applied 24 h after the addition of PGA1, quercetin-treated K562 cells showed a highly significant degree of heat resistance, which, however, was less pronounced than that exhibited at the same time by PGA1-treated cells kept in the absence of the flavonoid. At this time, hsp70 protein levels were similar in quercetin-treated and untreated cells. It remains to be established whether the moderate loss in thermotolerance shown at this time by quercetin-treated cells is a consequence of the antiproliferative activity after a 24-h treatment with the flavonoid or whether it reflects an accelerated turnover of the hsp70 protein.

Hyperthermia, alone or in combination with radiotherapy or chemotherapy, is now recognized and used as an effective form of treatment of certain types of cancer (13, 40, 41). Because thermotolerance has been one of the major limiting factors for the application of fractionated hyperthermia in cancer therapy, the attention of many authors has focused recently on the molecular mechanisms responsible for this phenomenon, in an attempt to identify factors that may lead to a differential response between normal and tumor tissue and to exploit a tumor-selective cytotoxicity for therapeutic gain. Hsps, in particular hsp70, have been implicated in thermotolerance, but a cause-and-effect relationship between hsp70 and heat resistance is still controversial. Microinjection of anti-hsp70 antibodies into rat embryo fibroblasts prevents the establishment of a thermotolerant phenotype in these cells (42). The same result could be achieved, also reducing the expression of hsp70, by competitively inhibiting the binding of HSF to the HSE in the promoter region of the hsp70 gene (43). Moreover, direct microinjection of human hsp70 into Chinese hamster ovary cells (43) or stable transfection of rat fibroblasts with a plasmid encoding for the human hsp70 gene (44) increases resistance to heating.

Fig. 9. Effect of quercetin on PGA1-induced thermotolerance. K562 cells (5 × 105 cells/ml) were treated with PGA1 (6 μg/ml; squares) or ethanol diluent (circles) in the presence (●) and absence (○) of 30 μM quercetin. Six h (A) or 24 h (B) after treatment, cells were subjected to a 45°C heat shock for 0, 15, 30, 45, or 60 min, and thermotolerance was tested by CSA. Differences between PGA1-treated cells kept in the absence or presence of quercetin were statistically significant at 30 and 45 min after heat challenge in A and 30, 45, and 60 min after heat challenge in B (P < 0.05).
The presence of a considerable amount of hsp27 in Chinese hamster cells transfected with the human hsp27 gene provided immediate tant phenotype to the stable transfectants (45). In Saccharomyces thermal resistance has been invoked for other hsps, hsp27 and hsplO4, splicing after heat damage (46, 47).

Under the conditions described, in K562 cells, PGA, was not found to induce hsp27 or hsp110 synthesis, whereas large amounts of hsp70 were detected up to 24 h after treatment. The fact that quercetin was able to suppress the establishment of a thermotolerant phenotype only during the period when hsp70 synthesis was inhibited supports strongly the hypothesis that this protein is involved in the regulation of thermotolerance in human cells.

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