Inhibition of heat shock factor activity prevents heat shock potentiation of glucocorticoid receptor-mediated gene expression

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Abstract Using mouse L929 cells stably transfected with a glucocorticoid receptor (GR)-responsive murine mammary tumor virus–chloramphenicol acetyltransferase (MMTV–CAT) reporter gene (LMCAT2 cells), we have shown that cellular stress (heat or chemical shock) can cause a dramatic increase in the levels of dexamethasone (Dex)-induced CAT gene expression. We refer to this response as the heat shock potentiation effect, or HSPE. As the cellular heat shock response also involves the activation of heat shock transcription factor (HSF), we have, in the present study, examined the role of HSF in the stress potentiation of GR by use of a flavonoid compound, quercetin, recently shown to selectively inhibit the stress response in a variety of human and murine cell lines. Analysis of the HSPE, as well as heat shock protein synthesis and activation of HSF during time-courses of recovery following heat shock, revealed a similar pattern for each response, with peak activities occurring about 16 h after stress. These data suggest a correlation between the activation of both GR and HSF in stressed cells. In L929 cells stably transfected with a CAT reporter plasmid under the control of the HSF-responsive *hsp70* promoter (LHSECAT cells), pretreatment with quercetin was found to cause a dose- and time-dependent inactivation of HSF activity following heat shock, but only when added before the stress event. In LMCAT2 cells, quercetin similarly inhibited both heat and chemical shock potentiation of Dex-induced GR activity. This activity of quercetin was not the result of post-transcriptional or general cytotoxic properties, as quercetin (1) did not significantly affect GR or HSF activities when added after the stress event, (2) did not reduce CAT gene expression as controlled by the constitutive SV40 early promoter, and (3) did not alter normal (non-stress), Dex-induced MMTV–CAT expression. Thus, quercetin appears to be an effective and selective inhibitor of HSF stress-induced activation and its ability to prevent the stress potentiation of GR suggests either a direct or indirect involvement by stress-activated HSF in this process, or the existence of a regulatory step common to both the heat shock and HSPE responses. © 1999 Harcourt Publishers Ltd

INTRODUCTION

The glucocorticoid receptor (GR) is a hormone-activated transcription factor that exists, in its unactivated state, as a large heteromeric complex containing the heat shock proteins Hsp90 and Hsp56 (Tsai and O'Malley 1994; Pratt and Toft 1997). The association of GR with heat shock proteins has led to reports demonstrating cross-talk

between the steroid and heat shock signal pathways. For example, the insect steroid hormone α -ecdysterone can induce the synthesis of small molecular weight HSPs in *Drosophila* (Ireland et al. 1982). Although there is little evidence in mammalian cells that glucocorticoid treatment will increase expression of HSPs, glucocorticoids have been shown to induce a stress-tolerant state in CHO cells (Fisher et al. 1986). In studies designed to test the effects of stress on the GR, we have found that heat and chemical shock (sodium arsenite) will cause the hormone-free mouse GR to translocate to the nucleus of L929 and CHO cells (Sanchez 1992; Shen et al. 1993), and similar results for human GR expressed in COS cells (Liu et al. 1995) and for the endogenous GR of rat liver (Matic 1995; Matic et al. 1995) have been reported.

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The ability of heat shock (HS) to affect the expression of glucocorticoid responsive genes has been demonstrated in a variety of ways. In L929 and CHO cells stably transfected with a GR-responsive reporter gene, we have shown that heat shock can partially activate the GR in the absence of hormone (Sanchez 1992; Shen et al. 1993), while combined stress and Dex treatment results in even greater GR-mediated gene expression than that seen in response to maximal hormone stimulation (Sanchez et al. 1994; Hu et al. 1996), a response we refer to as the heat shock potentiation effect (HSPE). More recently, it has been shown that GR-dependent transrepression of the collagenase promoter, a response involving GR interaction with AP-1, can be induced in the absence of hormone by heat shock treatment in both COS-7 and Hela cells (Liu et al. 1995). Similarly, heat shock has been found to mimic the ability of Dex to potentiate or repress Fc receptor expression (Fc γ R and Ia receptors, respectively) in murine macrophages (Sivo et al. 1996).

Although the above reports provide strong evidence for the regulation of GR function by cellular stress, the exact nature of this regulation is far from resolved. Indeed, a central assumption of our heat shock studies has been that induction of the cellular heat shock response, via activation of heat shock transcription factor (HSF), is required for the heat shock potentiation effect on the GR. Evidence to support this notion comes from Edwards et al. (1992) who found that the level of stress potentiation of progesterone receptor (PR)-mediated CAT gene expression in T47D cells correlated with the levels of *hsp70* induction. However, this type of correlation does not necessarily mean that HSF activation is involved in the HSPE, as the levels of HSF-mediated HSP induction may simply be a gauge for the magnitude of the stressful stimulus and that other factors in the cellular response are responsible for the HSPE. With this in mind, we have performed the present study in which we test if activation of HSF is required for the stress potentiation of the GR by use of a compound, quercetin, recently shown to inhibit the activities of HSF within intact cells. Quercetin is a flavonoid, widely distributed in plants, which can selectively inhibit the stress response in a variety of human and murine cell lines (Katengwa and Polla 1991; Hosokawa et al. 1992; Wei et al. 1994; Elia and Santoro 1994; Lee et al. 1994; Trautinger et al. 1995; Nagai et al. 1995; Sliutz et al. 1996), by a mechanism that appears to involve prevention of HSF1 hyperphosphorylation and inhibition of its binding to target DNA (Nagai et al. 1995). Our results demonstrate that inhibition of HSF activation by quercetin causes a concomitant loss of the stress potentiation effect on the GR, demonstrating a potential role for HSF in this process, and providing the first direct evidence for the intracellular convergence of the GR and HS signal pathways.

Fig 1 Determination of peak stress potentiation activity by pulse incubation of LMCAT2 cells with Dex during recovery from heat shock. LMCAT2 cells were subjected to heat shock at 43°C for 2 h (HS), or were not stressed (C). Immediately after heat shock, the cells were allowed to culture under non-stress conditions and 1 μ M Dex was added for 4 h intervals beginning at 0, 4, 8, 12, 16, 20, 24 and 44 h of recovery. A 4 h incubation with 1 μ M Dex was also performed on non-stressed cells (D). Lysates were prepared immediately after the 4 h incubation with hormone and analyzed for relative CAT enzyme activity as described under Materials and Methods. The results shown represent the means $\pm$ S.E.M. of 3–8 independent experiments.

MATERIALS AND METHODS

Materials

[3 H]Acetate (10.3 μ Ci/mmol) and [35 S]methionine ('Trans-label'; 1133 Ci/mmol) were obtained from ICN Radiochemicals. Autoradiography enhancer ('Amplify') was obtained from Amersham. Quercetin, DMSO, sodium arsenite, dexamethasone, G418 (Geneticin) antibiotic, acetyl CoA synthetase, acetyl Co-enzyme A, Tris, Hepes, EDTA, DMEM powdered medium were from Sigma Chemical Co. Iron-supplemented newborn calf serum and dialyzed fetal bovine serum were from Hyclone. Lipofectin was obtained from BRL.

In the p2500-CAT reporter, expression of chloramphenicol acetyltransferase (CAT) is controlled by the human *hsp70* promoter containing consensus heat shock elements (HSEs) known to be activated by the binding of heat shock factor (Dreano et al. 1986). The pMMTV-CAT plasmid contains the complete mouse mammary tumor virus-long terminal repeat promoter (MMTV-LTR) upstream of CAT (Danielsen et al. 1986). Hormonally driven expression of CAT by this reporter is controlled by glucocorticoid response elements (GREs) residing within the LTR region (Cato et al. 1986). The pGRE₂E1B-CAT minimal reporter is composed of two synthetic GREs derived from the tyrosine aminotransferase (TAT) promoter linked to the adenovirus E1B TATA sequence (Allgood et al. 1993). The pCAT-Control plasmid was obtained from Promega, Inc. In this plasmid, high-level, constitutive expression of the CAT reporter gene is controlled by the SV40 early promoter. In the pSV2neo

plasmid (Southern and Berg 1982), aminoglycoside phosphotransferase (*neo*) is also constitutively expressed from the SV40 early promoter.

Cell culture and stress/flavonoid treatment

The various CAT reporter-expressing cells lines were established as previously described (Sanchez et al. 1994; Ning and Sanchez 1996). Briefly, mouse L929 cells were co-transfected with pSV2neo and a two-fold excess of p2500-CAT (LHSECAT cells), pMMTV-CAT (LMCAT2 cells), pGRE₂E1B-CAT (LGEC cells) or pCAT-Control (LSVCAT cells), using lipofectin as carrier. This was followed by selection for stably transfected, cloned cell lines using G418 (Geneticin) antibiotic at 0.4 mg/ml. Once established, all cell lines were grown in an atmosphere of 5% CO₂ at 37°C in DMEM containing 0.2 mg/ml G418 and 10% iron-supplemented newborn calf serum. For all experiments, the newborn calf serum was stripped of endogenous steroids by extraction with dextran-coated charcoal. Most stress experiments were performed on cells that were at or near confluence, although similar results were obtained with sub-confluent cultures. Heat shock treatment was achieved by shifting replicate flasks to a second 5% CO₂ incubator set at 43°C. Typical duration of heat shock treatment was 1–2 h. Cells were also subjected to chemical shock by addition of 200 µM sodium arsenite to the medium. In the chemical shock experiments, the arsenite-treated and non-treated cells were incubated at 37°C for 2 h and were then washed with DMEM medium and allowed to recover. In the experiments involving quercetin, the drug was typically added to the culture media (stock concentration = 200 mM in DMSO) just prior to the stress event or for the indicated interval prior to hormone treatment. At the end of the stress or hormone treatments, the quercetin containing media was washed away, followed by continued culture as indicated for each experiment.

CAT assay

Measurement of CAT enzyme activity was performed according to the method of Nordeen et al. (1987) with minor modifications. Briefly, cell lysates were prepared by sequential freezing and thawing in 0.25 M Tris, 5 mM EDTA (pH 7.5) and centrifugation at 14 000 × *g*. Aliquots of lysate containing equal protein content were added to an enzymatic reaction mixture containing acetyl CoA synthetase, [³H]sodium acetate, coenzyme A and ATP. Radioactively labeled acetyl coenzyme A was first generated enzymatically from coenzyme A (CoA) and labeled acetate. Acetylation of chloramphenicol was then initiated by adding cell lysate containing CAT enzyme. The reaction was stopped by extraction with cold benzene

Fig 2 Relative rates of synthesis for *hsp70* and *hsp90* during recovery from heat shock. LMCAT2 cells were subjected to heat shock at 43°C for 2 h, or were left untreated (C). At the indicated time intervals during recovery (37°C), the cells were pulse-labeled with [³⁵S]methionine for 45 min. Whole cell lysates containing equal protein were separated by SDS-PAGE. Panel A: autoradiography of a typical gel. Panel B: quantitation of relative *hsp70* and *hsp90* synthesis rates by densitometric scanning of the bands. The results shown represent the means ± S.E.M. of three independent experiments.

and 75% of the organic phase was dried and counted. As the GRE- and HSE-containing promoters employed in this study have distinct basal and inducible activities, all data is represented as percent of maximum or the equivalent. In this way, the relative inhibitory effects of quercetin on HSF- and GR-mediated CAT expression can be more readily seen.

[³⁵S]Methionine labeling and preparation of whole cell extracts

In the experiment of Figure 2, LMCAT2 cells were heat shocked at 43°C for 2 h. At indicated intervals during recovery (37°C), the cells were pulse-labeled with [³⁵S]methionine for 45 min by removing the medium and replacing it with methionine-free medium containing 10% dialyzed calf serum and [³⁵S]methionine at a final concentration of 10 µCi/ml. All subsequent steps were carried out on ice (0–4°C). Cells were washed three times by pelleting and resuspension in Hank's buffered saline, followed by three cycles of freezing and thawing in 0.25 M Tris, 5 mM EDTA (pH 7.5) and centrifugation at 100 000 × *g*. The protein contents of these whole cell extracts were then determined by the BCA procedure of Pierce and the extracts were used immediately or were frozen at –80°C.

Gel electrophoresis and quantitative autoradiography

Samples were resolved by electrophoresis in 7% polyacrylamide SDS gels as described by Laemmli (1970). Following impregnation of the gels with an autoradiography enhancer ('Amplify'), the gels were dried under vacuum and mild heating (70°C) and exposed to Kodak XAR-5 X-ray film with an intensifying screen at -80°C. The relative amounts of newly synthesized *hsp70* and *hsp90* were calculated by densitometric scanning using the Bio-Rad Molecular Analyst software.

RESULTS

Correlation between the HSPE and heat shock responses

In initial work by our laboratory (Sanchez et al. 1994), we demonstrated the ability of stress to potentiate GR activity by subjecting LMCAT2 cells (L929 cells stably transfected with the pMMTV-CAT reporter) to heat shock and allowing the cells to recover in the presence of 1 μ M Dex for 20–24 h prior to assay for CAT activity. Under these conditions, the values for CAT obtained represented the total accumulated activity and, thus, could not be used to determine the time of peak activity for the HSPE response. As our first goal in the present project was to compare the HSPE response to the stress response (HSP induction and HSF activation), we have devised a method to measure peak HSPE activity by exposing LMCAT2 cells recovering from stress to short intervals (4 h) of Dex hormone treatment (Fig. 1). Under these conditions, the magnitudes of the stress potentiations were reduced over those seen in our prior report (Sanchez et

Fig 3 Relative levels of stress-induced HSF activity during recovery of LHSECAT cells from heat shock. LHSECAT cells were subjected to heat shock at 43°C for 2 h. At the indicated time interval during recovery (37°C), cell lysates were prepared and analyzed for CAT activity. The results shown represent the means $\pm$ S.E.M. of three independent experiments.

al. 1994) in which 20–24 h hormone exposures were used, but, nonetheless, a distinct temporal pattern for the HSPE was observed. Heat shock caused a depression of Dex-induced CAT activity during the early stages of recovery (0–4 h), and it was not until 8 h of recovery that the first evidence of the heat shock potentiation was observed. In addition, peak activity for the HSPE was rather broad (16–24 h) with values beginning their return to normal at 24 h of recovery.

In order to compare the HSPE response observed in Figure 1 with the response of these cells to stress, we assayed for both induction of HSPs and activation of HSF during recovery from heat shock. Measurement of HSP

Fig 4 Correlation between HSF activity and the heat shock potentiation of GR (HSPE) in cells subjected to dose-dependent chemical shock. LHSECAT and LMCAT2 cells were subjected to side-by-side treatment with increasing concentrations of sodium arsenite for 2 h, followed by addition of 1 μ M Dex to the LMCAT2 cells. Both cell lines were allowed to recover in the absence of arsenite for 20 h, followed by measurement of CAT activity (Panel A) and comparison of the HSF and HSPE responses (Panel B). HSPE values in the LMCAT2 cells were determined by subtracting CAT values obtained in response to hormone alone from those values derived from combined treatment (arsenite + Dex). A fit of the data using nonlinear regression is seen in Panel B (Spearman correlation coefficient $r = 0.9667$; $P < 0.0001$). The results shown represent the means $\pm$ S.E.M. of two independent experiments for LHSECAT cells and four independent experiments for LMCAT2 cells.

induction (Fig. 2) was performed by subjecting LMCAT2 cells to heat shock and pulse-labeling with [35 S]methionine at different times during recovery. Measurement of HSF activation (Fig. 3) was performed under similar conditions but using LHSECAT cells, made by stably transfecting L929 cells with an HSF-responsive reporter (p2500-CAT) under the control of the human *hsp70* promoter (Dreano et al. 1986). When compared with Figure 1, the results of Figures 2 and 3 demonstrate highly similar patterns, each with the following features: (i) a short period immediately after stress in which the activity is depressed (with the exception of *hsp70* induction), (ii) a relatively broad activity peak centered around 16 h of recovery, and (iii) return to normalcy beginning at 24 h. These observations suggest a strong correlation between GR and HSF activities in heat shocked cells. However, this correlation would only apply to one magnitude of stress, namely the 43°C, 2 h treatment employed. In order to more accurately measure correlation between these responses across a range of stress levels, we have performed the experiments seen in Figure 4 in which LMCAT2 and LHSECAT cells were subjected side-by-side to dose-dependent stress using sodium arsenite. As expected, the results demonstrate a strong correlation ($r = 0.9667$; $P < 0.0001$) between the HSF and HSPE activities in response to increasing concentrations of sodium arsenite.

Quercetin inhibits heat shock factor activation

As a variety of recent reports have demonstrated the ability of quercetin to selectively inhibit HSF activity under both in vitro and in vivo conditions (Katengwa and Polla 1991; Hosokawa et al. 1992; Wei et al. 1994; Elia and Santoro 1994; Lee et al. 1994; Trautinger et al. 1995; Nagai et al. 1995; Sliutz et al. 1996), we chose this drug as a means to test whether the HSPE response could be separated from stress-induced HSF activity. In initial experiments with LHSECAT cells, we measured HSF activity in response to heat shock (43°C, 2 h) in the constant presence of quercetin (during stress and during the recovery period). Under these conditions, viability of the cells was greatly reduced after 24 h of recovery. As an alternative approach, we incubated LHSECAT cells with increasing amounts of quercetin (0–200 μ M) only during heat shock (2 h), followed by recovery at 37°C in the absence of drug. In both non-stressed and heat shocked cells, this short-term treatment with quercetin (2 h) had no obvious effects on cell morphology or viability, while demonstrating a dose-dependent inhibition of heat shock-induced HSF activity (data not shown). Maximal inhibition of HSF activity was found to occur at 100 μ M drug. To further characterize the inhibitory effect of quercetin on HSF, we performed the experiments seen in Figure 5A and B. In

Fig 5 Quercetin inhibits stress-induced activation of HSF in LHSECAT cells. Panel A: When added before heat shock, quercetin inhibits HSF-induced CAT gene expression for up to 16 h of recovery. LHSECAT cells were subjected to heat shock at 43°C for 2 h in the presence or absence of quercetin (100 μ M). After removal of the quercetin, the cells were allowed to grow under nonstress conditions. At the indicated time intervals, cell lysates were prepared and analyzed for CAT activity. The results shown represent the means $\pm$ S.E.M. of two independent experiments. Panel B: When added after heat or chemical shock, quercetin does not inhibit HSF activity. LHSECAT cells were split into three groups. In the first group, LHSECAT cells were subjected to heat shock at 43°C for 2 h (HS), or were incubated with 200 μ M sodium arsenite for 2 h (CS), or were left unstressed (C). These cells were allowed to grow under non-stress conditions for 12 h. In the second group, LHSECAT cells were similarly stressed but in the presence of 100 μ M quercetin (Q-HS and Q-CS). For these cells the quercetin was washed away and the cells were cultured under non-stress conditions for 12 h. In the third group, quercetin (100 μ M) was added immediately after the stress event (HS-Q and CS-Q) and incubated for 2 h under non-stress conditions. After washing away of the quercetin, the cells were cultured for an additional 10 h. For all groups, cell lysates were prepared and analyzed for CAT activity. The results shown represent the means $\pm$ S.E.M. of three independent experiments.

Figure 5A, LHSECAT cells were treated with or without 100 μ M quercetin during heat shock followed by measurement of HSF activity over a time-course of recovery. As can be seen, the inhibitory effect of quercetin on heat

Fig 6 Quercetin has no effect on constitutively expressed or GR-induced CAT expression in non-stressed cells. Panel A: LSVCAT cells were left untreated (C), or were incubated with increasing concentrations of quercetin for 2 h followed by incubation for 12 h in the absence of quercetin. Panel B: LMCAT2 cells were left untreated (C), or were incubated with Dex (1 μM) for 12 h (D), or were incubated with increasing concentrations of quercetin for 2 h followed by 12 h treatment with Dex (1 μM). For each panel, cell lysates were prepared and analyzed for CAT activity. The results shown represent the means $\pm$ S.E.M. of three independent experiments.

shock-induced HSF activity is dependent on the time of recovery, as the inhibition persisted until 16 h of recovery, after which, the drug exhibited a stimulatory response (20 h) – a response consistent with a prior report (Elia and Santoro 1994).

Given the results of Figure 5A, we chose 12 h of recovery as the basis for further experimentation. Results from another laboratory have suggested that quercetin acts by preventing HSF activation, rather than by inhibiting the transcriptional enhancement properties of activated HSF (Hosokawa et al. 1992). In order to confirm this mechanism in our system, we performed the experiments of Figure 5B. The results demonstrate that quercetin can inhibit HSF activity induced by either heat or chemical shock (arsenite), when present concurrently with the

Fig 7 Quercetin inhibits heat shock potentiation of GR-mediated gene expression in LMCAT2 and LGEC cells, but only when added before stress. LMCAT2 cells (Panel A) and LGEC cells (Panel B) were subjected to a variety of hormone, stress and quercetin conditions. **C**: cells were cultured under normal conditions until harvested. **D**: cells were incubated with Dex (1 μM) for 12 h. **HS-D**: cells were first subjected to heat shock at 43°C for 2 h, followed by addition of 1 μM Dex for 12 h. **Q-HS-D**: cells were subjected to heat shock at 43°C for 2 h in the presence of quercetin (100 μM), the quercetin was washed away, and the cells were allowed to recover in the presence of Dex (1 μM) for 12 h. **HS-Q-D**: cells were first subjected to heat shock at 43°C for 2 h followed by addition of quercetin (100 μM) and Dex (1 μM) at 37°C for 2 h. The quercetin was washed away and the cells were allowed to recover in the continuing presence of Dex for another 10 h. The results of each panel represent the means $\pm$ S.E.M. of three independent experiments.

stress event, but that this inhibition does not occur when quercetin is added after stress. Thus, although the precise mechanism of quercetin action remains unknown, quercetin appears to be an agent that can selectively inhibit one or more of the early events by which HSF is activated in response to stress.

Analysis of the potential nonspecific effects of quercetin

Based on the results of Figure 5B, in which quercetin was shown to have no effect on the transcriptional enhancement activity of activated HSF, it is likely that quercetin, as

Fig 8 Quercetin inhibits chemical shock potentiation of the GR. LMCAT2 cells were subjected to the following conditions: **C**, cells were cultured under normal conditions until harvested; **D**, cells were incubated with Dex (1 μ M) for 12 h; **CS-D**, cells were first subjected to chemical shock (200 μ M sodium arsenite for 2 h), followed by addition of 1 μ M Dex for 12 h; **Q-CS-D**, cells were subjected to chemical shock for 2 h in the presence of quercetin (100 μ M), the quercetin was washed away, and the cells were allowed to recover in the presence of Dex (1 μ M) for 12 h. The results represent the means $\pm$ S.E.M. of three independent experiments.

herein employed, has little or no effect on general gene expression or post-transcriptional events. In order to confirm these properties, the effect of quercetin on CAT gene expression as controlled by the constitutive SV40 early promoter was determined by use of L929 cells stably transfected with the pCAT-Control plasmid (LSVCAT cells). In the experiments of Figure 6A, LSVCAT cells were treated with increasing concentrations of quercetin for 2 h, followed by incubation in the absence of quercetin for 12 h. The results show that CAT activities were unaltered in response to quercetin treatment. Although the above suggests no non-specific effects of quercetin on generalized gene expression, it remained a possibility that quercetin could affect the actions of select transcription factors in addition to HSF. In particular, we were concerned that the actions of the GR could be directly affected by quercetin treatment. To test this possibility, the effects of quercetin on Dex-induced CAT activity in non-stressed LMCAT2 cells were measured (Fig. 6B). The results show that 2 h incubations with quercetin up to 100 μ M had no effect on hormone-induced GR activity. Taken as a whole, the results of Figures 5 and 6 demonstrate that the conditions of quercetin treatment employed in this study can be used to selectively inhibit activation of HSF, while having no significant effects on general gene transcription or the actions of GR in unstressed cells.

Quercetin inhibits heat and chemical shock potentiation of the GR

The results of Figure 5 demonstrated that the inhibitory effect of quercetin on HSF could be reliably measured at

12 h of recovery. As the HSPE response could also be seen at 12 h of recovery in the LMCAT2 cells (Fig. 1), we chose this recovery time-point as the appropriate time at which to measure the effects of quercetin treatment on the stress potentiation of the GR. In the experiments of Figure 7A, the effect of quercetin on the HSPE response in LMCAT2 cells was measured at 12 h of recovery from heat shock. The results show a strong heat shock-induced increase in GR-mediated CAT activity and a complete inhibition of this potentiation when quercetin was added before the stress event. In contrast, quercetin only had a small inhibitory effect on the HSPE when added after the heat shock treatment. Thus, the inhibitory effect of quercetin on the HSPE closely parallels its effects on HSF activity (Fig. 5B), suggesting that the inhibitory effect of quercetin on the HSPE is primarily due to inactivation of HSF or to inhibition of a factor common to both responses.

As the MMTV promoter employed in the LMCAT2 cells is $\approx$ 1.2 kb in size, we could not exclude the possibility that the actions of quercetin in stressed cells occurred via a DNA-binding factor that acted to inhibit the actions of GR. To test this idea, L929 cells stably-transfected with the pGRE₂E1B-CAT minimal promoter (LGEC cells) were subjected to similar conditions of quercetin and heat shock treatment (Fig. 7B). In this promoter, expression of CAT is controlled by two tandemly linked synthetic GREs and a TATA box (Allgood et al. 1993). The results again demonstrate a complete inhibition of the HSPE response when quercetin was added before, but not after, the stress event. As a final test of the actions of quercetin on the HSPE, the ability of quercetin to inhibit chemical shock potentiation of the GR was measured in Figure 8. Consistent with our other findings, a complete inhibition of the potentiation effect was again observed.

DISCUSSION

In this study, we have provided evidence for a linkage between the heat shock signal pathway and the pathway controlling stress potentiation of the GR. Our observations to support this conclusion are of two types. First, we demonstrate a correlation in our cells between the heat shock potentiation effect on the GR (HSPE) and HSF activity, as measured by HSP induction and CAT reporter response. Second, we show that the flavonoid compound quercetin can be used to selectively prevent the activation of HSF in stressed cells and that this treatment results in loss of the HSPE response, while having no effect on GR signaling under nonstress conditions.

Our finding of a correlation between the HSPE and HSF activities is consistent with the observations of Edwards et al. (1992), in which a qualitative correlation was seen in human T47D cells between levels of *hsp70* induction in response to heat shock and potentiation by

Fig 9 Model for quercetin inhibition of HSF and GR activities in stressed cells. Based on the results of this study, in which we have used quercetin (Q) to show that inhibition of HSF activity can prevent the heat shock potentiation effect (HSPE) on the GR, we propose that inhibition of the HSPE response by quercetin can occur by one of two general mechanisms. In the first mechanism (A), the HSPE is prevented as a consequence of HSF inhibition, presumably through the actions of quercetin on an enzyme (kinase?) involved in stress-activation of HSF. Under this scenario, the HSPE response would, therefore, be due to a HSF-induced gene product (X) that may, for example, act as a stress-induced co-activator of the GR. In the second mechanism (B), the effects of quercetin on the HSF and HSPE responses are independent of each other. In this case, inhibition of each by quercetin is due to the existence of a common target involved in the stress regulation of both HSF and the GR. (For further details, see the Discussion).

heat shock of progesterone receptor (PR)-mediated CAT gene expression. The authors also showed that the maximal effects of heat shock on *hsp70* synthesis and potentiation of PR activity occurred immediately at the end of the heat shock treatment, with both activities declining during the recovery period. In our L929 cells, maximal *hsp70* synthesis (not to mention *hsp90* synthesis and HSF activity) and the HSPE remain correlated, but occur during the recovery period (see Figs 1–3). Although technical considerations may ultimately explain this discrepancy, it is also possible that it is simply due to cell-type specific

factors. In this report and others (Edwards et al. 1992; Sanchez et al. 1994; Hu et al. 1996), we have consistently observed that chemical shock using sodium arsenite results in higher levels of stress potentiation of the GR than does heat shock, a result also seen for the PR of T47D cells (Edwards et al. 1992). We also show that arsenite is a more efficient inducer of HSF activity (see Fig. 5B). Thus, our results and those of others are consistent with a model in which the magnitude of stress potentiation of GR is largely determined by the overall activity of HSF.

In mammalian cells, regulation of HSF in response to stress has been extensively studied, although a clear understanding of all steps involved has yet to be achieved [see Morimoto et al. (1996) for review]. It is generally accepted that the first step in stress activation of HSF is the dissociation of inactive HSF/HSP complexes due to the recruitment of the HSPs to act as chaperones in the refolding of denatured proteins (Anathan et al. 1986). At present, it is controversial as to whether *hsp70* or *hsp90* is the principal negative regulator of HSF activity (Morimoto et al. 1992; Zou et al. 1998; Ali et al. 1998; Duina et al. 1998). The second step appears to be assembly of HSF into trimers and this ability is derived from multiple leucine zipper domains (Rabindran et al. 1993). Translocation of HSF to the nucleus and interaction with heat shock response elements in the promoter regions of HSP genes then occurs. Full-blown transcriptional enhancement activity has been shown to require phosphorylation of HSF on serine residues (Sorger 1991), although the presumably stress-activated kinase(s) involved has yet to be identified. Based on this model of HSF actions, how then can we explain the actions of quercetin to prevent the heat shock potentiation of the GR? A possible explanation can be found in the data of Figures 5B and 7, in which we demonstrate that the inhibitory effect of quercetin on the HSF and HSPE activities is only seen when the drug is added before, but not after, the stress event. This pattern suggests that the event that triggers the HSF/HSPE activities is not reversible by quercetin treatment. Thus, it is highly unlikely that quercetin acts by inhibiting the transcription enhancement activities of HSF or the GR once they are bound to their cognate response elements. Instead, our data argue that the effect of quercetin on HSF and, consequently, on the HSPE, must be at an early stage of the HSF signal pathway.

In one of the earliest studies of this kind (Hosokawa et al. 1990), treatment of Hela and COLO 320DM cells with quercetin prevented elevated synthesis of all the major HSPs (Hsp110, Hsp90, Hsp70, Hsp47, Hsp40 and Hsp28) in response to heat shock or sodium arsenite. This inhibitory effect of quercetin has been shown to be at the level of transcription, as demonstrated by both Northern blot analysis (Hosokawa et al. 1990) and RNase protection assay (Hosokawa et al. 1992) using probes derived from the human *hsp70* DNA. Although reports exist for an inhibitory effect of quercetin on RNA polymerases in permeabilized human fibroblast cells (Nose 1984) or intact *E. coli* (Ono et al. 1990), it is unlikely that quercetin is acting by this mechanism in intact mammalian cells. In Hela cells, RNase protection assays did not demonstrate the expected pattern of shorter *hsp70* mRNA fragments that inhibition of RNA polymerase II should have generated (Hosokawa et al. 1992). In our system, no effect of

quercetin on RNA polymerase II was evident, as this drug had no effect on GR-mediated CAT expression under non-stress conditions, and had little effect on HSF- or GR-mediated CAT expression when added after the stress event. Thus, quercetin appears to inhibit HSP synthesis at the transcriptional level by a mechanism that likely involves a direct or indirect inhibition of HSF.

More recent studies have provided additional insight into this mechanism. In COS-7 and COLO 320DM cells, quercetin not only inhibited heat shock-induced expression of CAT under the control of the *hsp70* promoter, but also inhibited formation of HSF-HSE complexes following treatment of intact cells with quercetin prior to heat shock (Hosokawa et al. 1992). This laboratory also showed that quercetin could inhibit HSF-HSE complex formation under in vitro conditions in which cytoplasmic extracts containing HSF were activated by heat or treatment with urea (Hosokawa et al. 1992). It is interesting to note that in vitro activation of HSF DNA-binding function was not inhibited when quercetin was added after heat or urea treatment (Hosokawa et al. 1992), suggesting again that quercetin acts by preventing an early step in the activation of HSF. As previously mentioned, two early steps in the HSF activation process are stress-induced trimerization and hyperphosphorylation. Using gel filtration and chemical cross-linking approaches, quercetin was found to have no effect in Hela cells on the extent of HSF trimer formation in response to heat shock (Nagai et al. 1995). Thus, this early stage in HSF activation appears to proceed normally in the presence of quercetin. In the same study (Nagai et al. 1995), quercetin was found to selectively reduce both the amount of HSF that is constitutively phosphorylated in non-stressed cells and the amount of HSF that is hyperphosphorylated in response to heat shock. As the amount of overall HSF protein was also reduced as a consequence of quercetin pretreatment, it was proposed that prevention of HSF hyperphosphorylation by quercetin may lead to rapid degradation of HSF by proteases in heat shocked cells (Nagai et al. 1995). Taken as a whole, these observations point to a model of quercetin action in which heat shock-induced phosphorylation of HSF is prevented shortly after the trimerization step, resulting in down-regulation of HSF protein and, as a consequence, inhibition of all down-stream events in the heat shock signal mechanism.

Based on the above, we currently have two working hypotheses, illustrated in Figure 9, by which to explain the effects of quercetin and the role of HSF on activity of GR in stressed cells. In the first model (Fig. 9A), we postulate that the stress potentiation of the GR is mediated by an HSF-induced gene product, expression of which is inhibited by quercetin. This gene product may or may not be a member of the heat shock family of proteins. For this

reason, we have simply designated it as 'X'. Under this hypothesis, it is interesting to speculate that this gene product could act as a co-activator which would function to increase the stimulatory effect of hormone-activated GR on Pol II activity. This mechanism is at least plausible, as we have earlier shown that the potentiation effect on the GR can be seen for hormone-bound GR that is already driven to the nucleus prior to the stress treatment, demonstrating that the potentiation effect principally acts on the transcription enhancement function of the GR (Sanchez et al. 1994). We have also shown [Sanchez et al. (1994) and this work, Fig. 7B] that the stress potentiation of GR can occur for a CAT reporter under the control of a synthetic, minimal promoter composed solely of GREs, showing that the potentiation effect cannot be the result of cooperativity between the GR and another DNA-binding protein. Thus, if a HSF-induced protein is involved in a direct protein-protein interaction with the GR, we would predict that it would meet the criteria of a stress-induced, steroid receptor co-activator. Of course, it is equally possible that this HSF-induced product serves some other role, such as directly or indirectly modifying the GR, perhaps through phosphorylation. An effect of this product to somehow increase retention of the GR within the nucleus would also serve to explain the heat shock potentiation phenomenon. However, the latter mechanism is unlikely, as we have shown that heat shock does not increase the amount of GR within the nucleus of hormone-treated cells (Sanchez et al. 1994).

In the second model (Fig. 9B), we consider the possibility that the actions of quercetin on the HSF and HSPE activities are mediated by a target that is common to each pathway. In this case, quercetin may be acting by inhibiting an enzyme (kinase?) that directly or indirectly causes independent activation of HSF and potentiation of the GR. Under this model, it would be possible to separate the HSF and HSPE responses. We are currently pursuing alternative means by which to inactivate HSF in stressed cells and the effects these treatments have on the stress potentiation of GR.

Each of the above models requires a protein or factor that serves as the target for quercetin actions, but the identity of this target is far from resolved. Quercetin has been reported to have a variety of biochemical effects, such as inhibition of Na⁺/K⁺ ATPases (Kuriki and Racker 1976), inhibition of adenylate cyclase (Graziani and Chayoth 1979) and inhibition of a various other protein kinases (End et al. 1987). It is generally accepted that most of quercetin actions can be explained by its ability to preferentially bind ATP-binding sites. With regard to Na⁺/K⁺ ATPases or adenylate cyclase, it is clear that the effect of quercetin on HSF is not mediated by these enzymes, as inhibition by quercetin of HSF activation to the DNA-binding state has been demonstrated in

cell-free lysates devoid of these enzymes (Hosokawa et al. 1992). It is of particular interest that quercetin has been reported to inhibit protein kinase C (PKC) activity (Gschwendt et al. 1983), as heat shock has been shown to increase PKC activity (Wooten 1991), and as the potent PKC activator, tetradecanoyl phorbol acetate (TPA), can also cause activation of HSF (Auger et al. 1992). Moreover, the PKC inhibitors, staurosporine and H7, have been shown to inhibit expression of the HSP genes, *hsp28*, *hsp70* and *hsp83* (Katengwa and Polla 1991; Kim et al. 1993). Although it is tempting to conclude that the primary target of quercetin's inhibitory actions on HSF is PKC, proof of this relationship will require genetic approaches to the identification of the kinase(s) involved in stress-mediated HSF phosphorylation.

Taken as a whole, the results of this study point to a necessary role by the heat shock signal pathway in the process by which cellular stress causes a super-activation of GR-mediated reporter gene expression. Experiments are underway, using genetic and cell-free systems, to determine whether HSF activity per se, or some other stage of heat shock signaling, is required for the potentiation of GR. Meanwhile, the recent demonstrations of a role for heat shock in the regulation of GR-mediated expression of endogenous genes [collagenase (Liu et al. 1995); FcγRI and Ia receptors of macrophages (Sivo et al. 1996)] underscores the physiological significance of our observations and suggests that further studies on the molecular intersection of the GR and HS pathways may prove beneficial in the development of new or alternative therapies.

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REFERENCES

- Ali A, Bharadwaj S, Carroll RO and Ovsenek N (1998) Hsp90 interacts with and regulates the activity of heat shock factor 1 in *Xenopus* oocytes. *Mol Cell Biol* **18**: 4949–4960.
- Allgood VE, Oakley RH and Cidlowski JA (1993) Modulation by vitamin B₆ of glucocorticoid receptor-mediated gene expression requires transcription factors in addition to the glucocorticoid receptor. *J Biol Chem* **268**: 20870–20876.
- Anathan JA, Goldberg AL and Voellmy R (1986) Abnormal proteins serve as eukaryotic stress signals and trigger the activation of heat shock genes. *Science* **232**: 252–254.
- Auger EA, Giaccia AJ, Brown JM and Hahn GM (1992) Evidence that protein kinase C is involved in the activation of heat shock

- transcription factor by ionizing radiation. In: *Hyperthermic Oncology*, edited by Gerner E. Tucson: p. 99.
- Cato ACB, Miksicek R, Schutz G, Arnemann J and Beato M (1986) The hormone regulatory element of mouse mammary tumour virus mediates progesterone induction. *EMBO J* 5: 2237–2240.
- Danielsen M, Northrop JP and Ringold GM (1986) The mouse glucocorticoid receptor: mapping of functional domains by cloning, sequencing and expression of wild-type and mutant receptor proteins. *EMBO J* 5: 2513–2522.
- Dreano M, Brochot J, Myers A, Cheng-Meyer C, Rungger C, Voellmy R and Bromley P (1986) High-level, heat-regulated synthesis of proteins in eukaryotic cells. *Gene* 49: 1–8.
- Duina AA, Kalton HM and Garber RF (1998) Requirement for hsp90 and cyp-40-type cyclophilin in negative regulation of heat shock response. *J Biol Chem* 273: 18974–18978.
- Edwards DP, Estes PA, Fadok VA, Bona BJ, Onate S, Nordeen SK and Welch WJ (1992) Heat shock alters the composition of heteromeric steroid receptor complexes and enhances receptor activity in vivo. *Biochemistry* 31: 2482–2491.
- Elia G and Santoro G (1994) Effect of quercetin on prostaglandin A1-induced heat shock response in human cells. *Annals New York Acad Sci* 744: 323–325.
- End D, Look RA, Shaffer NL, Balles EA and Persico FJ (1987) Non-selective inhibition of mammalian protein kinases by flavonoids in vitro. *Res Commun Chem Pathol Pharmacol* 56: 75–86.
- Fisher GA, Anderson RL and Hahn GM (1986) Glucocorticoid-induced heat resistance in mammalian cells. *J Cell Phys* 128: 127–132.
- Graziani Y and Chayoth R (1979) Regulation of cyclic AMP level and synthesis of DNA, RNA and protein by quercetin in Ehrlich ascites tumor cells. *Biochem Pharmacol* 28: 397–403.
- Gschwendt M, Horn F, Kittstein W and Marks F (1983) Inhibition of the calcium- and phospholipid-dependent protein kinase activity from mouse brain cytosol by quercetin. *Biochem Biophys Res Commun* 117: 444–447.
- Hosokawa N, Hirayoshi K, Nakai A, Hosokawa Y, Marui N, Yoshida M, Sakai T, Nishino H, Aoike A, Kawai K and Nagata K (1990) Flavonoids inhibit the expression of heat shock proteins. *Cell Struct Funct* 15: 393–401.
- Hosokawa N, Hirayoshi K, Kudo H, Takechi H, Aoike A, Kawai K and Nagata K (1992) Inhibition of the activation of heat shock factor in vivo and in vitro by flavonoids. *Mol Cell Biol* 12: 3490–3498.
- Hu JL, Guan X-J and Sanchez ER (1996) Enhancement of glucocorticoid receptor-mediated gene expression by cellular stress: evidence for the involvement of a heat shock-initiated factor or process during recovery from stress. *Cell Stress & Chaperones* 1: 197–205.
- Ireland RC, Berger EM, Sirotkin K, Yund MA, Osterbur D and Fristrom J (1982) Ecdysterone induces the transcription of four heat shock genes in *Drosophila* S3 cells and imaginal discs. *Dev Biol* 93: 498–507.
- Katengwa S and Polla BS (1991) Flavonoids, but not protein kinase C inhibitors, prevent stress protein synthesis during erythrophagocytosis. *Biochem Biophys Res Commun* 180: 308–314.
- Kim SH, Kim JH, Erdos G and Lee YJ (1993) Effect of staurosporine on suppression of heat shock gene expression and thermotolerance development in HT-29 cells. *Biochem Biophys Res Commun* 193: 759–763.
- Kuriki Y and Racker E (1976) Inhibition of (Na⁺, K⁺) adenosine triphosphatase and its partial reactions by quercetin. *Biochemistry* 15: 4951–4956.
- Laemmli UK (1970) Cleavage of structural proteins during the assembly of the heat of bacteriophage T4. *Nature* 227: 680–685.
- Lee JL, Erdos G, Hou Z, Kim SH, Kim JH, Cho JM and Corry PM (1994) Mechanism of quercetin-induced suppression and delay of heat shock gene expression and thermotolerance development in HT-29 cells. *Mol Cell Biochem* 137: 141–154.
- Liu W, Hillmann AG and Harmon JM (1995) Hormone-independent repression of the AP-1-inducible collagenase promoter activity by glucocorticoid receptors. *Mol Cell Biol* 15: 1005–1013.
- Matic G (1995) A cross-talk between glucocorticoid receptor-mediated signal transduction pathway and heat shock response. *Jugoslav Med Biochem* 14: 89–102.
- Matic G, Kipic J, Ristic B, Dundjerski J and Trajkovic D (1995) Hyperthermic stress modulates the functions of rat liver glucocorticoid receptor. *Cell Biol Int* 19: 203–213.
- Morimoto RI, Sarge KD and Abravaya K (1992) Transcriptional regulation of heat shock genes. *J Biol Chem* 267: 21987–21990.
- Morimoto RI, Kroeger PE and Cotto JJ (1996) The transcriptional regulation of heat shock genes: a plethora of heat shock factors and regulatory conditions. In: *Stress-inducible Cellular Responses*, edited by Feige U, Morimoto RI, Yahara I and Polla B. Basel: Birkhauser Verlag: p. 139–163.
- Nagai N, Nakai A and Nagata K (1995) Quercetin suppresses heat shock response by down regulation of HSF1. *Biochem Biophys Res Commun* 208: 1099–1105.
- Ning Y-M and Sanchez ER (1996) In vivo evidence for the generation of a glucocorticoid receptor-hsp90 complex incapable of binding hormone by the calmodulin antagonist phenoxylbenzamine. *Mol Endocrinol* 10: 14–23.
- Nordeen SK, Green PP III and Fowlkes DM (1987) A rapid, sensitive and inexpensive assay for chloramphenicol acetyltransferase. *DNA* 6: 173–178.
- Nose K (1984) Inhibition by flavonoids of RNA synthesis in permeable WI-38 cells and of transcription by RNA polymerase II. *Biochem Pharmacol* 33: 3823–3827.
- Ono K, Nakane H, Fukushima M, Chermann J and Barre-Sinoussi F (1990) Differential inhibitory effects of various flavonoids on the activities of reverse transcriptase and cellular DNA and RNA polymerases. *Eur J Biochem* 190: 469–476.
- Pratt WB and Toft DO (1997) Steroid receptor interactions with heat shock protein and immunophilin chaperones. *Endo Rev* 18: 306–360.
- Rabindran SK, Haroun RI, Clos J, Wisniewski J and Wu C (1993) Regulation of heat shock factor trimer formation: role of conserved leucine zipper. *Science* 259: 230–234.
- Sanchez ER (1992) Heat shock induces translocation to the nucleus of the unliganded glucocorticoid receptor. *J Biol Chem* 267: 17–20.
- Sanchez ER, Hu JL, Zhong SJ, Shen P, Green MJ and Housley PR (1994) Potentiation of glucocorticoid receptor mediated gene expression by heat and chemical shock. *Mol Endocrinol* 8: 408–421.
- Shen P, Xie Z-J, Li H and Sanchez ER (1993) Glucocorticoid receptor conversion to high affinity nuclear binding and transcription enhancement activity in Chinese hamster ovary cells subjected to heat and chemical stress. *J Steroid Biochem Mol Biol* 47: 55–64.
- Sivo J, Harmon JM and Vogel SN (1996) Heat shock mimics glucocorticoid effects on IFN- γ -induced Fc γ RI and Ia messenger RNA expression in mouse peritoneal macrophages. *J Immunol* 156: 3450–3454.
- Sliutz G, Karlseder J, Tempfer C, Orel L, Holzer G and Simon MM (1996) Drug resistance against gemcitabine and topotecan mediated by constitutive hsp70 overexpression in vitro: implication of quercetin as sensitiser in chemotherapy. *Br J Cancer* 74: 172–177.
- Sorger PK (1991) Heat shock factor and the heat shock response. *Cell* 65: 363–366.

- Southern PJ and Berg P (1982) Transformation of mammalian cells to antibiotic resistance with a bacterial gene under control of the SV40 early region promoter. *J Mol Biol Appl Gen* 1: 327–341.
- Trautinger F, Kindas-Mugge I, Barlan B, Neuner P and Knobler RM (1995) 72-kD heat shock protein is a mediator of resistance to ultraviolet B light. *J Invest Dermatol* 105: 160–162.
- Tsai M-J and O'Malley BW (1994) Molecular mechanisms of action of steroid/thyroid receptor superfamily members. *Ann Rev Biochem* 63: 451–486.
- Wei YQ, Zhao W, Kariya Y, Fukata H, Teshigawara K and Uchida A (1994) Induction of apoptosis by quercetin: involvement of heat shock protein. *Cancer Res* 54: 4952–4957.
- Wooten MW (1991) Alterations in protein kinase C type III- α during heat shock of rat embryo fibroblasts. *Exp Cell Res* 193: 274–278.
- Zou J, Guo Y, Guettouche T, Smith DF and Voellmy R (1998) Repression of heat shock transcription factor HSF1 activation by hsp90 (hsp90 complex) that forms a stress-sensitive complex with HSF1. *Cell* 94: 471–480.