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Modulation of the Heat-induced Activation of Mitogen-activated Protein (MAP) Kinase by Quercetin

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ABSTRACT. Effects of quercetin, a bioflavonoid compound, on heat-induced activation of mitogen-activated protein (MAP) kinase in rat hepatoma (H4) cells were examined. Quercetin decreased cell viability and induced DNA fragmentation in heat-shocked H4 cells. MAP kinase in heat-shocked cells was activated and reached a peak at 1 hr after the heat shock, and then gradually decreased. Quercetin inhibited the heat-induced activation of MAP kinase observed at 1 hr after heat shock, but markedly stimulated MAP kinase activity at 4 hr after heat shock. Thus, quercetin modulated the heat-induced activation of MAP kinase in a biphasic manner. Present observations indicate that quercetin modulates protein phosphorylation, especially that controled by MAP kinase, in early events of heat shock response. BIOCHEM PHARMACOL **56**;9:1151–1155, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. quercetin; heat shock proteins; MAP kinase; apoptosis; protein phosphatase 2A

Heat shock provokes numerous alterations in the metabolism of cells [1]. Reversible protein phosphorylation is one of the major mechanisms by which protein activity is controled in the processes of heat shock responses. Heat shock causes phosphorylation of the initiation factor 2 and dephosphorylation of ribosomal protein S6 and initiation factor 4B and 4F in HeLa cells [2]. RNA polymerase II C-terminal domains are phosphorylated in heat-shocked HeLa cells [3]. The alterations in the phosphorylation state of these proteins belonging to the transcriptional and translational apparatus cause the inhibition of protein synthesis in heat-shocked cells. On the other hand, cells exposed to heat shock transiently synthesize a group of proteins called heat shock proteins (HSPs§) [1]. Some HSPs (90 kDa HSP and 27 kDa HSP) have been shown to be phosphorylated in response to heat shock [4, 5]. Transcription of heat shock genes is regulated by the *cis*-acting heat shock element in the promoter region and the *trans*-acting HSF [1]. The transcriptional activity of HSF is also regulated by phosphorylation [6–9]. Protein phosphorylation is catalyzed by protein kinases and reversed by protein phosphatases. MAP kinase is activated by heat shock *in vivo* and *in vitro* [10, 11]. MAP kinase is considered to be the upstream kinase which phosphorylates 27 kDa HSP [12], the RNA polymerase II C-terminal domain [3], and HSF1 [9]. This evidence indicates that MAP kinase is a key mediator in intracellular signal transduction in heat shock responses.

Quercetin, a bioflavonoid compound, is known as a hyperthermic sensitizer [13]. Quercetin induces apoptosis in some cell lines [14] and has been reported to inhibit the activation of HSF1 [15, 16] and the production of HSPs in heat-shocked cells [17]. Inhibition of HSP production suppresses the acquisition of thermotolerance [18]. Based on these observations, it is believed that quercetin induces cell death in heat-shocked cells by the suppression of HSP production. Although quercetin is also known to be a nonspecific inhibitor of protein kinases [19], the effects of quercetin on protein phosphorylation shortly after heat shock have not yet been elucidated. In the present study, we examined the effects of quercetin on the MAP kinase activation observed shortly after heat shock.

MATERIALS AND METHODS

Cell Culture and Heat Shock Conditions

H4 cells were grown at 37° on tissue culture dishes with Swims-77S medium supplemented with 5% fetal bovine serum and 5% horse serum. At 1 hr before experimentation, the incubation medium was changed to Krebs–Ringer–HEPES buffer, pH 7.4, containing 132 mM NaCl, 4.8 mM

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§ *Abbreviations:* HSPs, heat shock proteins; MAP kinase, mitogen-activated protein kinase; HSF, heat shock factor; PMSF, phenylmethylsulfonyl fluoride; and PP2A, protein phosphatase 2A.

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KCl, 2.4 mM $MgCl_2$, 0.1 mM EGTA, 1 mM $CaCl_2$, 20 mM HEPES, 10 mM glucose, and 0.1% of BSA. For heat shock, 100-mm culture dishes were immersed in a water bath for 10 min at 45°. The cells (10^6 cells) were processed for assays at 0 to 4 hr incubation at 37° after heat shock. Cell viability was determined by the trypan blue dye exclusion test.

DNA Fragmentation Assay

At the end of incubation, cells were scraped in 1 mL of PBS and pelleted by centrifugation at 200 g for 5 min. Cell pellets were lysed and incubated in 0.2 mL of 10 mM Tris buffer, pH 8.0, containing 10 mM of EDTA, 150 mM of NaCl, 0.4% of SDS, and 0.5 mg/mL of proteinase K for 1 hr at 65°. DNA was extracted with an equal volume of phenol:chloroform (1:1). After centrifugation, the upper DNA-containing phase was mixed with 0.1 volume of 3 M sodium acetate and 2.5 volume of ethanol to precipitate DNA. The DNA was pelleted by centrifugation and resuspended in 0.1 mL of 10 mM Tris buffer, pH 8.0, containing 1 mM EDTA and 1 mg/mL of RNase A for 1 hr at 37°. The DNA was extracted again with phenol:chloroform and ethanol precipitation. Extracted DNA was electrophoresed on a 1.5% agarose gel for 2 hr at 100 V, and visualized by ethidium bromide staining and examination under UV illumination.

Assay of HSP Synthesis

Cells were metabolically labeled with 50 μCi/dish of [^{35}S]-methionine in 5 mL of Krebs–Ringer–HEPES buffer at 37° for 1 or 4 hr after the heat treatment [20]. After washing with cold PBS, the cells were lysed in 0.5 mL of 50 mM HEPES buffer, pH 7.4 containing 1% of Triton X-100, 0.1% of SDS, 5 mM EDTA, 1 mM of EGTA, 1 mM PMSF, and 1 mg/mL of leupeptin. The cell lysates prepared from equal numbers of the cells were analyzed by SDS-PAGE. Synthesized proteins were identified by autoradiography.

HSP70 was assayed by immunoblot analysis. Equal amounts of protein for each sample were separated by SDS-PAGE and blotted to PVDF membrane. The membrane was incubated with monoclonal anti-HSP70 antibody (Santa Cruz). The bound antibody was detected by horseradish peroxidase-linked secondary antibodies. Immunostaining was done in the solution (15 mg of 4-chloro-1-naphthol dissolved in 5 mL of cold methanol with 25 mL of 20 mM Tris–HCl-buffered saline, pH 7.5 and 5 μL of hydrogen peroxide) for 30 min.

Mini Purification and the Assay of MAP Kinase

Mini purification of MAP kinase was done by the method previously described [21]. Briefly, cells were disrupted by sonication in 0.8 mL of buffer A (25 mM Tris buffer, pH 7.5, containing 25 mM NaCl, 40 mM *p*-nitrophenylphosphate, 1 mM sodium orthovanadate, 2 mM EGTA, 1 mM dithithreitol, and 0.2 mM PMSF on ice, followed by centrifugation at 20,000 *g* for 5 min. The supernatant fraction was immediately mixed with 0.2 mL of packed phenyl-Sepharose in a 1.5 mL microcentrifuge tube. After brief centrifugation, the unbounded material was removed. The phenyl-Sepharose was then successively washed with buffer A containing 10 and 30% ethylene glycol. A fraction eluted by 45% ethylene glycol was used as partially purified MAP kinase (0.5 mg/mL protein). The MAP kinase assay was performed by the method reported by Sturgill *et al.* [22].

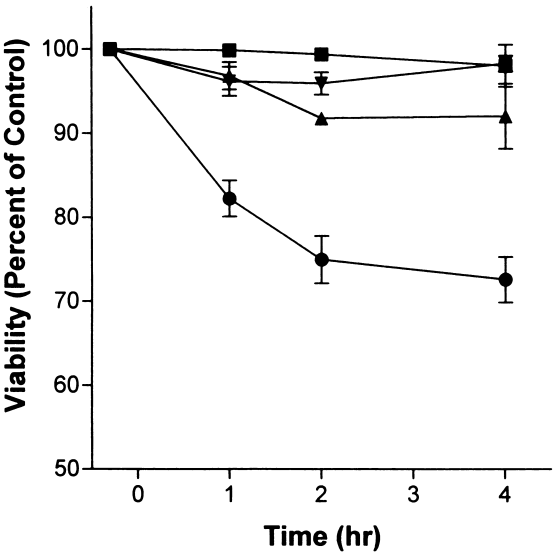

FIG. 1. Effects of heat shock and quercetin on H4 cell viability. H4 cells were incubated for 10 min at 45° and then for 1 to 4 hr at 37°. Cell viability was assayed by the trypan blue dye exclusion test. Data are presented as the means ± SEM of 3 independent experiments. Control (■), heat shock (▼), 0.1 mM of quercetin (▲), 0.1 mM of quercetin + heat shock (●).

RESULTS

Effects of Heat Shock and Quercetin on H4 Cell Viability

We treated H4 cells at 45° for 10 min and then incubated them at 37° for 0–4 hr. As shown in Fig. 1, the viability of heat-shocked cells was comparable to that of control cells. Although treatment of cells with 0.1 mM quercetin alone slightly decreased cell viability, more than 90% of the cells survived for the 4-hr incubation at 37°. In the presence of 0.1 mM quercetin, the viability of heat-shocked cells rapidly decreased to 75% of control cells within the 2-hr incubation at 37° after heat shock. Although the trypan blue dye exclusion test is an insensitive method for the assay of cell death, present data showed that quercetin induced cell death in heat-shocked H4 cells shortly after heat shock.

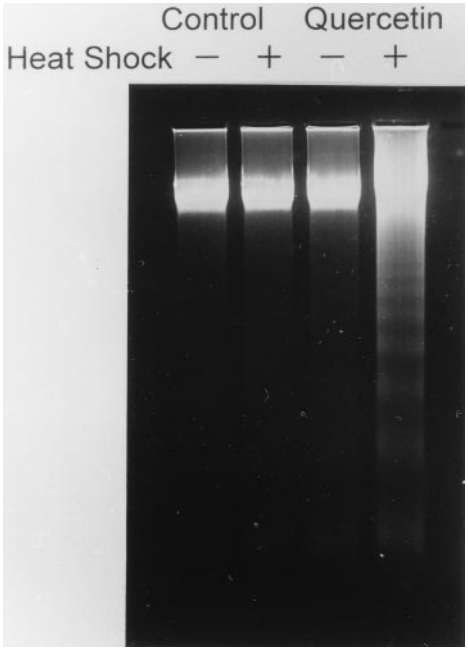

FIG. 2. Effects of heat shock and quercetin on DNA fragmentation in H4 cells. H4 cells were incubated for 10 min at 45° and then for 1 hr at 37°. At the end of incubation, DNA was extracted and analyzed by 1.5% agarose gel electrophoresis.

Effects of Heat Shock and Quercetin on DNA Fragmentation in H4 Cells

Quercetin induces apoptosis accompanied by DNA fragmentation in several cell lines [14]. Therefore, we examined whether quercetin induced apoptosis with DNA fragmentation in H4 cells. The assay was done at 1 hr after heat shock. As shown in Fig. 2, neither heat shock nor quercetin alone induced DNA fragmentation. In the presence of 0.1 mM of quercetin, heat shock induced DNA fragmentation.

Effects of Quercetin on the Production of HSPs in H4 Cells

Production of HSPs was assayed by metabolic labeling of proteins by [^{35}S]-methionine and immunoblot analysis. The production of 4 HSPs (110 kDa, 90 kDa, 70 kDa, and 27 kDa) was identified at 4 hr after heat shock. Quercetin inhibited the synthesis of all HSPs (Fig. 3, panel A). Immunoblot analysis of HSP70 showed that HSP70 was produced at 4 hr, but not at 1 hr after heat shock (Fig. 3, panel B).

Effects of Heat Shock and Quercetin on the Activity of MAP Kinase

MAP kinase plays a central role in the protein phosphorylation-regulating heat shock responses [3, 9–12]. Therefore, we next examined the effects of quercetin on heat-induced activation of MAP kinase. As shown in Fig. 4, MAP kinase activity in heat-shocked cells increased and reached its peak at 1-hr incubation at 37° after heat shock, and then gradually decreased. In the presence of 0.1 mM of quercetin, heat-induced activation of MAP kinase at 1 hr after heat shock was inhibited. Interestingly, quercetin markedly stimulated MAP kinase activity at 4 hr after heat shock. Thus, quercetin modulated the heat-induced activation of MAP kinase in a biphasic manner.

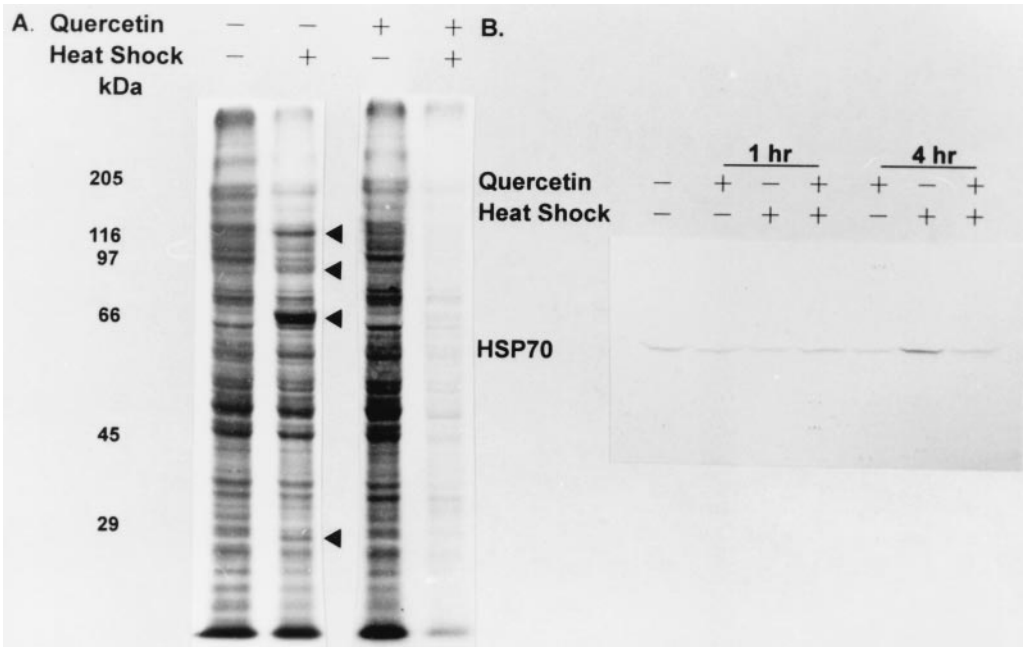

FIG. 3. Effects of quercetin on the production of HSPs in H4 cells. H4 cells were incubated for 10 min at 45° and then for 1 or 4 hr at 37°. (A) Cells were harvested at 4 hr after heat shock. HSPs were assayed by metabolic labeling of proteins with [^{35}S]-methionine. The labeled proteins were separated by SDS-PAGE and analyzed by autoradiography. (B) Cells were harvested at 1 and 4 hr after heat shock. HSP70 was assayed by immunoblot analysis.

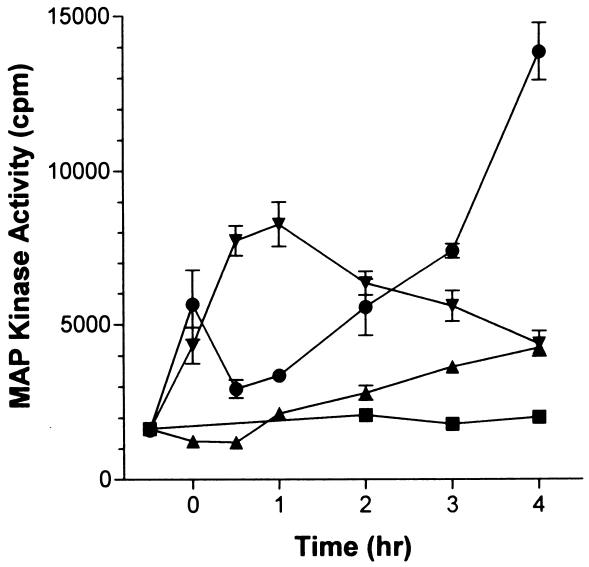

FIG. 4. Effects of heat shock and quercetin on the activity of MAP kinase. H4 cells were incubated for 10 min at 45° and then for 0 to 4 hr at 37°. Cells were harvested at indicated times and processed for mini purification and assay of MAP kinase activity. Data are presented as the means ± SEM of 5 independent experiments. Control (■), heat shock (▼), 0.1 mM of quercetin (▲), 0.1 mM of quercetin + heat shock (●).

Effects of Okadaic Acid on the Inhibition by Quercetin of the Heat-induced Activation of MAP Kinase

MAP kinase is activated by the phosphorylations of the threonine and tyrosine residues. MAP kinase is phosphorylated by the dual-specific protein kinases and dephosphorylated by PP2A [23]. PP2A activity is considered to be a determinant for the inactivation of MAP kinase in the cytosol. Therefore, we examined the effects of okadaic acid (a specific inhibitor of PP2A) on the inhibitory action of quercetin on heat-induced MAP kinase activation. As shown in Fig. 5, okadaic acid enhanced heat-induced MAP kinase activation and restored the inhibitory action of quercetin on heat-induced MAP kinase activation at 1 hr after heat shock. At 4 hr after heat shock, okadaic acid further enhanced the activation of MAP kinase induced by heat shock in the presence of quercetin (data not shown).

DISCUSSION

Quercetin was reported to be a hyperthermic sensitizer in HeLa cells [13]. Quercetin has various biological activities: the inhibition of cultured cell growth [13], inhibitory effects on glycolysis [24], macromolecule synthesis [25], and the activity of protein kinases [19] and ATPases [26]. Furthermore, quercetin was reported to inhibit HSF activity and HSP synthesis at the transcriptional level [15–17]. Because the synthesis of HSPs is a major event in heat shock responses, the inhibition of HSP synthesis has been believed to be one of the mechanisms of cell death induced by quercetin [14–18]. However, the present study demonstrated that quercetin induced cell death and DNA fragmentation in heat-shocked H4 cells before HSPs were produced. The present data suggest that quercetin acts on early events before HSP synthesis in heat-shocked H4 cells.

Changes in the phosphorylation state of intracellular proteins are considered to play an important role in early events in heat shock responses as described in the introduction. MAP kinase is an upstream kinase in the intracellular signal transduction of heat shock responses [3, 9–12]. The data reported herein showed that MAP kinase was activated by heat shock and reached a peak at 1 hr after heat shock, and then gradually decreased (Fig. 4). In these cells, HSPs were not synthesized at 1 hr after heat shock (Fig. 3, panel B). Quercetin inhibited the heat-induced activation of MAP kinase observed at 1 hr after heat shock, and markedly stimulated MAP kinase at 4 hr after heat shock (Fig. 4). Thus, quercetin modulated the heat-induced activation of MAP kinase in a biphasic manner. These observations indicate that quercetin modulates protein phosphorylation, especially that controled by MAP kinase, in early events of the heat shock response.

The mechanism of the biphasic effect of quercetin is not clear. Quercetin had no direct effects on MAP kinase activity. Okadaic acid, a specific PP2A inhibitor, restored the inhibitory action of quercetin on the heat-induced activation of MAP kinase (Fig. 5). Quercetin may inhibit heat-induced MAP kinase activation through the activation of PP2A.

Recently, it was reported that HSF was phosphorylated by MAP kinase, which decreased the transcriptional activity [9]. MAP kinase was reported to act as a negative

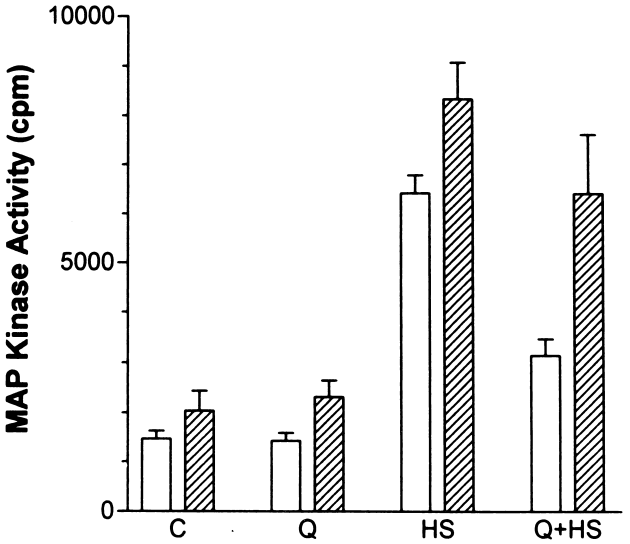

FIG. 5. Effects of okadaic acid on the inhibition by quercetin of heat-induced activation of MAP kinase. H4 cells were incubated for 10 min at 45° and then for 1 hr at 37° in the absence (open column) or presence of 0.1 μM of okadaic acid (shaded column). Cells were harvested and processed for mini purification and assay of MAP kinase activity. Data are presented as the means ± SEM of 3 independent experiments. C, control; Q, 0.1 mM of quercetin; HS, heat shock; Q + HS, 0.1 mM of quercetin + heat shock.

regulator of the heat shock responses in NIH3T3 cells [27]. In the present experiments (Fig. 3, panel B and Fig. 4), the production of HSPs was not observed when MAP kinase was activated in heat-shocked H4 cells (at 1 hr after heat shock). The production of HSPs could be observed after the decline in MAP kinase activity (at 4 hr after heat shock). Quercetin stimulated MAP kinase markedly at 4 hr after heat shock and inhibited the production of HSPs in heat-shocked H4 cells. These and earlier reported observations suggest that activation of MAP kinase in heat-shocked cells may inhibit the production of HSPs through the phosphorylation of HSF1.

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