Preheating Accelerates Mitogen-activated Protein (MAP) Kinase Inactivation Post-heat Shock via a Heat Shock Protein 70-mediated Increase in Phosphorylated MAP Kinase Phosphatase-1*

Received for publication, September 1, 2004, and in revised form, January 13, 2005
Published, JBC Papers in Press, January 26, 2005, DOI 10.1074/jbc.M410059200

Kyoung-Hee Lee, Choong-Taek Lee, Young Whan Kim, Sung Koo Han, Young-Soo Shim, and Chul-Gyu Yoo‡

From the Division of Pulmonary and Critical Care Medicine, Department of Internal Medicine, and the Lung Institute, Seoul National University College of Medicine, Seoul 110-799, Korea and the Clinical Research Institute, Seoul National University Hospital, Seoul 110-744, Korea

Heat shock (HS) activates mitogen-activated protein (MAP) kinases. Although prior exposure to nonlethal HS makes cells refractory to the lethal effect of a subsequent HS, it is unclear whether this also occurs in MAP kinase activation. This study was undertaken to evaluate the effect of a heat pretreatment on MAP kinase activation by a subsequent HS and to elucidate its possible mechanism. Preheating did not make BEAS-2B cells refractory to extracellular signal-regulated protein kinase (ERK) and c-Jun N-terminal kinase (JNK) activation by a second HS but accelerated their inactivation after HS. The rapid inactivation of ERK and JNK was dependent on de novo protein synthesis and associated with the up-regulation of heat shock protein 70 (HSP70). Moreover, the inhibition of phosphatase activity reversed this rapid inactivation. MAP kinase phosphatase-1 (MKP-1) expression was increased by HS, and the presence of its phosphorylated form (p-MKP-1) correlated with the observed rapid ERK and JNK inactivation. Blocking induction of p-MKP-1 with antisense MKP-1 oligonucleotides suppressed the rapid inactivation of ERK and JNK in preheated cells. HSP70 overexpression caused the early phosphorylation of MKP-1. Moreover, MKP-1 phosphorylation and the rapid inactivation of ERK were inhibited by blocking HSP70 induction in preheated cells. In addition, MKP-1 was insubilized by HS, and HSP70 associated physically with MKP-1, suggesting that a chaperone effect of HSP70 might have caused the early phosphorylation of MKP-1. These results indicate that preheating accelerated MAP kinase inactivation after a second HS and that this is related to a HSP70-mediated increase in p-MKP-1.

The mitogen-activated protein (MAP)1 kinase cascade is an intracellular signaling module ubiquitous among eukaryotes.

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‡ To whom correspondence should be addressed: Division of Pulmonary and Critical Care Medicine, Dept. of Internal Medicine, Seoul National University Hospital, 28 Yongon-dong, Chongno-gu, Seoul 110-744, Korea. Tel.: 82-2-2072-3760; Fax: 82-2-762-9662; E-mail: cgyno@snu.ac.kr.
† The abbreviations used are: MAP, mitogen-activated protein; HS, heat shock; HSP, heat shock protein; MKP-1, MAP kinase phosphatase-1; p-MKP-1, phosphorylated MKP-1; ERK, extracellular signal-regulated protein kinase; p-ERK, phosphorylated ERK; JNK, c-Jun N-terminal kinase; CHX, cycloheximide; PBS, phosphate-buffered saline; OA, okadaic acid; SV, sodium orthovanadate; MG132, N-carboxymethyl-Leu-Leu-Leu-leucinal; Ad-HSP70, adenovirus vectors expressing HSP70; Ad-β-gal, adenovirus expressing β-galactosidase; Ad-AS-HSP70, adenovirus vectors expressing HSP70 antisense RNA; PP2A, protein phosphatase 2A; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase.
HSP70-mediated Phosphorylation of MKP-1 in MAPK Inactivation

MATERIALS AND METHODS

Cell Culture—Normal human bronchial epithelial cells, BEAS-2B, were maintained as a monolayer in a keratinocyte growth medium (Invitrogen). NIH3T3 cells were maintained in Dulbecco's minimal essential medium containing 10% fetal bovine serum, 60 μg/ml penicillin, and 100 μg/ml streptomycin at 37 °C under 5% CO₂.

Reagents—Rabbit polyclonal anti-JNK1 and anti-MKP-1 antibodies, goat polyclonal anti-actin antibody, mouse monoclonal anti-HSP70 antibody, and recombinant glutathione S-transferase-c-Jun were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Rabbit polyclonal anti-ERK and anti-phosphorylated ERK antibodies were from Cell Signaling (Beverly, MA). Goat anti-rabbit secondary antibody conjugated with horseradish peroxidase was from Santa Cruz Biotechnology. Lipofectamine 2000 was purchased from Invitrogen. Cycloheximide (CHX), okadaic acid, sodium orthovanadate, and protein G-Sepharose beads were obtained from Sigma. The ECL kit was purchased from Amersham Biosciences. Protease inhibitors were purchased from Roche Applied Science. The proteasome inhibitor N-carbonyloxy-Leu-Leu-Leu-leucinal (MG132) was obtained from the Peptide Institute (Osaka, Japan), and ATP was supplied by Cell Signaling (Beverly, MA). The PP2A assay kit was obtained from Promega (Madison, WI).

HS Treatment—In all experiments, HS response was induced by incubating cells in a water bath at 43 °C. After initial HS treatment, the culture medium was removed and replaced with fresh medium. Cells were allowed to recover in a 5% CO₂ incubator at 37 °C for the indicated times.

Western Blot Analysis—Twenty micrograms of protein were resolved by 10% SDS-PAGE and transferred to nitrocellulose membranes. The membranes were blocked with 5% skim milk, PBS, 0.1% Tween 20 for 1 h before being incubated overnight at 4 °C with primary antibodies diluted 1:1,000 in 5% milk skim, PBS, 0.1% Tween 20. The membranes were then washed three times in 1× PBS, 0.1% Tween 20 and incubated with goat anti-rabbit horseradish peroxidase-conjugated antibodies diluted 1:1,000 in 5% skim milk, PBS, 0.1% Tween 20 for 1 h. After successive washes, the membranes were developed using an ECL kit.

Kinase Assay—JNK activities were assessed using an in vitro kinase assay as previously described (24). In brief, JNK1 was immunoprecipitated with anti-JNK1 antibody. The immunoprecipitates were incubated at 30 °C for 30 min in a kinase buffer containing 0.5 μg of glutathione S-transferase-c-Jun and 0.2 mM ATP. The kinase reaction products were subjected to SDS-PAGE in 10% gels followed by transfer to a nitrocellulose membrane and analysis by Western blot.

Transduction of Adenoviruses—Recombinant adenovirus vectors encoding HSP70 sense or antisense RNA were kindly provided by Dr. Michael Sherman (Department of Biochemistry, Boston University Medical School) and Dr. Dick Mosser (Department of Molecular Biology and Genetics, University of Guelph, Guelph, Ontario, Canada). A recombinant adenovirus expressing β-galactosidase (Ad-β-gal) was used as a control virus. Cells were plated at a concentration of 1 × 10⁵ cells/well in a 6-well tissue culture plate. After overnight incubation, cells were transduced at multiplicities of infection of 50 by adenovirus vector in a keratinocyte growth medium for 2 h with gentle shaking and then washed with PBS and incubated with medium at 37 °C, 5% CO₂ until use. After 24 h, the cells were used in the experiments indicated.

Antisense Experiments—BEAS-2B cells were transiently transfected with antisense (5'-ccACCTTCCATGACCAtg-3') or sense (5'-ccATG-GTCATGGAAGTggtc-3') phosphorothioate-modified oligonucleotides of the MKP-1 gene as reported previously (25) (phosphorothioate modifications are shown by lowercase letters). Oligonucleotides were used in a final concentration of 0.05 μg/12-well plate. Transfection was performed using Lipofectamine 2000 according to the manufacturer's specifications. After 2 h, the cells were used in the experiments indicated.

RESULTS

Heat Pretreatment Did Not Prevent HS-induced MAP Kinase Activation but Accelerated Its Inactivation—To determine the impact of HS on MAP kinases, we first analyzed the time-dependent activation of MAP kinases by HS. Phosphorylated ERK (p-ERK) was hardly detectable in the basal state. p-ERK started to increase at 30 min after HS and increased further with prolonged exposure to HS up to 2 h (Fig. 1A). On the other hand, HS did not affect the total ERK expression level (Fig. 1A). The time course of HS-induced JNK activation was similar to that of ERK activation (Fig. 1A). The same amounts of JNK1 in immunoprecipitates were confirmed by Western blot analysis (Fig. 1A). We next explored whether the HS-induced activation of MAP kinases is suppressed in cells rendered thermo-tolerant. The time-dependent effect of preheating on the activation of these MAP kinases by a subsequent HS was investigated. ERK and JNK activities were both increased by exposure to 2-h HS, and this was unaffected by preheating, thus suggesting that preheating did not block the HS-induced...
activation of MAP kinase (Fig. 1B). Interestingly, the rate of ERK and JNK inactivation during the recovery period after HS was more rapid in preheated cells than that in nonpreheated cells (Fig. 1B and C). In contrast, the increase in phosphorylated MEK-1 was sustained for 4 h after HS in preheated cells (Fig. 1B). Thus, it appears unlikely that the rapid inactivation of upstream activators is responsible for the short duration of MAP kinase activation in preheated cells. Taken together, events occurring downstream of MAP kinase activation may be required for the accelerated inactivation of MAP kinase in preheated cells.

Rapid MAP Kinase Inactivation by Preheating Required de Novo Protein Synthesis—We evaluated whether rapid inactivation of MAP kinase in preheated cells is dependent on de novo protein synthesis. As shown in Fig. 1, ERK and JNK activities were higher than at base line at 4 h after HS in nonpreheated cells, whereas at this time they had returned to basal levels in preheated cells. Thus, we evaluated the duration of MAP kinase activation by comparing MAP kinase activities at 4 h after HS. Increased ERK and JNK activities at 4 h after HS were unaffected by CHX treatment in nonpreheated cells (Fig. 2A). However, in preheated cells, whereas ERK and JNK activities returned to base line at 4 h after HS, their activities remained high in the presence of CHX (Fig. 2A). The inhibition of de novo protein synthesis by CHX treatment was confirmed by the suppression of HSP70 induction by HS (Fig. 2A). Moreover, treatment with CHX alone did not activate ERK (Fig. 2B).

These findings indicate that de novo protein synthesis is required for the accelerated inactivation of MAP kinase in preheated cells.

HSP70 Induction Was Associated with the Rapid Inactivation of MAP Kinase by Preheating—HSPs are representative proteins induced by HS and have been suggested to be responsible for thermotolerance. Thus, HSP70 accumulation during the preheating may be associated with accelerated MAP kinase inactivation. Indeed, preheating enhanced HSP70 induction by the subsequent HS (Fig. 2A). In order to determine the role of HSP70, we first evaluated the effect of HSP70 overexpression on the rate of MAP kinase inactivation. To express HSP70 at high levels, we infected cells with adenovirus constructs encoding HSP70 sense RNA (Ad-AS-HSP70) or Ad-β-gal. The overexpression of HSP70 was confirmed by immunoblotting (Fig. 3A). The increase in p-ERK was sustained for 4 h after HS in Ad-β-gal-infected cells (Fig. 3A). In contrast, p-ERK began to decrease at 2 h after HS and further decreased at 4 h after HS in Ad-HSP70-infected cells (Fig. 3A), thus suggesting the role of HSP70 in the accelerated inactivation of MAP kinase. To further confirm the relationship between HSP70 and the rate of MAP kinase inactivation, HSP70-overexpressing cells were made by stably transfecting a
plasmid encoding HSP70 into NIH3T3 cells. p-ERK was up-regulated 30 min after HS to a similar degree in both mock-transfected and HSP70-overexpressing cells (Fig. 3B). Moreover, the HS-induced p-ERK increase was sustained for 2 h after HS in mock-transfected cells; in contrast, it returned to base line 2 h after HS in HSP70-overexpressing cells (Fig. 3B). The overexpression of HSP70 was confirmed by Western blotting (Fig. 3B). To confirm the association between HSP70 and ERK inactivation, we evaluated the effect of blocking HSP70 induction on the time-dependent ERK inactivation. HSP70 induction is necessary for rapid MAP kinase inactivation. To confirm that PP2A activity was stable during the HS and recovery processes, we measured the phosphatase activity of PP2A. The phosphatase activity of PP2A did not change either in nonpreheated or preheated cells (Fig. 5B). MKP-1 was not detected at base line or at 2 h after HS. However, MKP-1 was detected when cells were allowed to recover for 4 h after HS in nonpreheated and preheated cells, thus suggesting that a certain recovery time is required for MKP-1 induction (Fig. 5C, lanes 3, 5, 7, and 9). MKP-1 was detected as one band in nonpreheated cells and in preheated cells that were not allowed to recover (Fig. 5C, lanes 3 and 5). In contrast, in cells that were allowed to recover for 4 or 24 h after preheating, MKP-1 contained slowly migrating bands in addition to rapidly migrating bands (Fig. 5C, lanes 7 and 9). The increased HSP70 expression persisted up to 24 h after HS (Fig. 5C). These slowly migrating bands were confirmed to be p-MKP-1 by immunoprecipitation with anti-MKP-1 antibody followed by immunoblotting with anti-phosphoserine antibody (Fig. 5D). ERK and JNK activities were reduced to base line 4 h after HS when preheated cells were allowed recovery times of 4 or 24 h, which coincided with p-MKP-1 detection (Fig. 5C, lanes 7 and 9). Thus, a recovery period of more than 4 h after preheating is required to accelerate MAP kinase inactivation and the phosphorylation of MKP-1. OA or SV pretreatment suppressed the increased rate of MAP kinase inactivation by preheating (Fig. 4B). Both unphosphorylated and phosphorylated MKP-1 induction was inhibited in preheated cells upon OA or SV treatment (Fig. 5E). These results suggest that p-MKP-1 is probably associated with accelerated MAP kinase inactivation.

Phosphorylated MKP-1 Was Responsible for Rapid MAP Kinase Inactivation—MKP-1 has been reported to be a labile protein that is normally degraded via the ubiquitin/proteasome pathway. It is also known that its phosphorylation reduces its ubiquitination and degradation (29, 30). Thus, if p-MKP-1 induction is responsible for accelerated MAP kinase inactivation by preheating, then the up-regulation of MKP-1 by proteasome inhibition should also accelerate this inactivation. p-MKP-1 expression was up-regulated 6 h after treating with MG132, without affecting ERK or JNK activity (Fig. 6, A and B). Moreover, MG132 pretreatment did not affect the HS-induced activations of ERK and JNK (Fig. 6A). However, increases in the activities of ERK and JNK 4 h after HS were completely abrogated by MG132 pretreatment (Fig. 6B), suggesting that up-regulated p-MKP-1 is involved in accelerated MAP kinase inactivation after HS. Interestingly, MG132-induced MKP-1 was decreased by HS (Fig. 6A). To confirm that the preheating-induced acceleration of MAP kinase inactivation is associated with MKP-1 induction, we investigated whether the preheating-induced acceleration of MAP kinase inactivation was suppressed by blocking MKP-1 induction. To block MKP-1 induction, we used MKP-1 antisense oligonucleotide, which (partially) blocked MKP-1 induction (Fig. 6C). High levels of ERK and JNK activity were present 4 h after HS in preheated cells transfected with MKP-1 antisense oligonucleotides (Fig. 6C). This implies that the preheating-induced acceleration of
MAP kinase inactivation was reversed by blocking MKP-1 induction. In contrast, transfection with MKP-1 sense oligonucleotide affected neither MKP-1 induction nor the preheating-induced acceleration of MAP kinase inactivation (Fig. 6C).

These results indicate that MKP-1 induction is responsible for accelerated MAP kinase inactivation.

Up-regulation of HSP70 by Preheating Was Involved in the Early Phosphorylation of MKP-1—We previously demonstrated that heat denaturation is responsible for the loss of IκB kinase solubility and activity (31). In Fig. 6A, MG132-induced MKP-1 was rapidly reduced after HS, suggesting MKP-1 denaturation by HS. In order to evaluate whether MKP-1 was denatured by heat treatment, its expression was measured in soluble and in insoluble extracts after heat treatment. Basal MKP-1 expression was not observed in either soluble or insoluble extracts. MKP-1 was detected only in insoluble extracts 2 h after HS in nonpreheated cells but was present in both soluble and insoluble extracts in preheated cells (Fig. 7A). This indicates that MKP-1 induced by HS was rapidly denatured, and the preheating partially blocked its denaturation by HS. When heat-shocked cells were allowed to recover at 37 °C for 4 h, unphosphorylated or phosphorylated MKP-1 was detected in soluble extracts from nonpreheated or preheated cells, respectively (Fig. 7A). This suggests that preheating may be associated with the presence of p-MKP-1. To determine the association between HSP70 induction and the phosphorylation of MKP-1, we compared the time kinetics of HS-induced HSP70 and MKP-1 induction. MKP-1 induction started 1 h after HS and persisted for 4 h in both nonpreheated and preheated cells. HS-induced MKP-1 was mainly present in the unphosphorylated form in nonpreheated cells but in the phosphorylated form in preheated cells (Fig. 7B). Moreover, the level of HSP70 expression was higher in preheated cells than that in nonpreheated cells 0, 1, 2, or 4 h after HS (Fig. 7B). To confirm the association between HSP70 induction and the phosphorylation of MKP-1, we evaluated the time dependence of HS-induced MKP-1 phosphorylation in HSP70-overexpressing NIH3T3 cells. HS-induced unphosphorylated MKP-1 was detected in mock-transfected cells, whereas it caused the phosphorylation of MKP-1 in HSP70-overexpressing cells (Fig. 7C). In addition, p-MKP-1 appeared earlier in HSP70-overexpressing BEAS-2B cells than in control adenovirus-infected cells (Fig. 7D). To evaluate whether HSP70 elevation is necessary for MKP-1 phosphorylation, we investigated the effect of blocking HSP70 induction on the phosphorylation of MKP-1 in preheated cells. When HSP70 induction was inhibited by adenovirus vectors expressing HSP70 antisense RNA, preheating did not lead to MKP-1 phosphorylation, and the preheating-induced rapid inactiva-
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A

|     | MG132 | - | + | + | + | - | - |
|-----|-------|---|---|---|---|---|---|
| HS  | -  | 1/2 | 1 | 1/2 | 1 | 2 | (h) |

**Fig. 6.** p-MKP-1 was responsible for rapid MAP kinase inactivation. A, MG132 up-regulated p-MKP-1, and it did not affect HS-induced increase in ERK and JNK activities. BEAS-2B cells were incubated with 10 μM of MG132 for 6 h, and then cells were exposed to 43°C for 0.5, 1, or 2 h. B, the up-regulation of p-MKP-1 by MG132 accelerated MAP kinase inactivation. BEAS-2B cells were incubated with MG132 (10 μM) for 6 h and then were heat-shocked for 2 h and allowed to recover for 4 h (HS2/4). C, blocking MKP-1 induction abolished accelerated MAP kinase inactivation. BEAS-2B cells were transfected with MKP-1 sense (S-MKP-1) or antisense oligonucleotide (AS-MKP-1) using Lipofectamine 2000. After 3 h, cells were exposed to 1 h of HS and allowed to recover for 4 h (Pre-HS1/4). Cells were heat-shocked for 2 h and allowed to recover for 4 h (HS2/4). MKP-1 and actin expressions were assayed by Western blot analysis. ERK and JNK activities were assayed by Western blot analysis for its phosphorylated form (p-ERK) and by immune complex kinase assays, respectively. Results are representative of three separate experiments.

In this study, preheating did not make cells refractory to MAP kinase activation by a subsequent HS. This contrasts with the findings of a previous study, in which the pretreatment of NIH3T3 cells with mild heat shock suppressed UV-induced JNK activation (32). Thus, the heat desensitization of MAP kinase activation might be stimulus-specific. It was interesting that preheating shortened the duration of the subsequent HS-induced activation of MAP kinases. This implies that preheating accelerated the rate of MAP kinase inactivation, which raises the question of how this might occur. One possible mechanism may be a preheating-induced alteration of upstream regulators. However, because MEK-1 was normally activated by HS in preheated cells, rapid MAP kinase inactivation by preheating is unlikely to be caused by the down-regulation of upstream signaling molecules of MAP kinase.

Thermotolerance is induced by preheating followed by a recovery period before a subsequent heat challenge (18, 19). In this study, a recovery period of more than 4 h after preheating was also required for accelerated MAP kinase inactivation. This suggests the possible role of de novo protein synthesis during recovery from HS. We also found that pretreatment with CHX abolished the effect of preheating on the accelerated inactivation of MAP kinases. Thus, it seems likely that preheating enhanced the rate of MAP kinase inactivation through the transcriptional induction of some protein(s) that negatively regulates MAP kinases. HS is characterized by the induction of HSPs. Indeed, preheating enhanced HSP70 induction by HS in the present study. The role of HSP in thermotolerance has been suggested in many previous studies (20–23). HSP70 overexpression attenuated the proteotoxicity of cellular ATP depletion (33). Furthermore, the overexpression of HSP70 prevented apoptosis in response to a variety of stresses by suppressing JNK activation (32, 34, 35). Therefore, it is likely that HSP70 accumulated after preheating acts as a repressor of the MAP kinase pathways. As shown in preheated cells, the overexpression of HSP70 did not make cells refractory to MAP kinase activation by a subsequent HS. This result is consistent with other observations indicating that permanent cell lines constitutively expressing HSP70 genes respond normally or even slightly better to the heat activation of JNK than control cells (36–38). In addition, the overexpression of HSP by gene transfection did not cause desensitization, and the inhibition of HSP synthesis did not prevent desensitization to the activation of p38 by heat (39). In contrast to the inability of HSP70 overexpression to make cells refractory to a second HS-induced MAP kinase activation, the rate of MAP kinase inactivation was faster in HSP70-overexpressed cells than that in mock-transfected cells. Moreover, we showed that blocking HSP70 induction delayed ERK inactivation. These results suggest that HSP70 is implicated in the preheating-induced acceleration of MAP kinase inactivation.

The activation of MAP kinase requires dual phosphorylation on its threonine and tyrosine residues, and the dephosphorylation of these residues terminates such activation (26–28). In this study, the short duration of MAP kinase activation in preheated cells was not found to be associated with the suppression of its upstream activator. Therefore, one possibility is that it was rapidly dephosphorylated by phosphatase(s). A number of phosphatases can dephosphorylate ERK both in vitro and in vivo (40). For example, PP2A can inactivate both MEK and ERK (41, 42). The dual specificity phosphatase is another phosphatase involved in MAP kinase signaling. Dual specificity phosphatase, MKP-1, is an emerging subclass of the protein tyrosine phosphatase gene superfamily, which appears to selectively dephosphorylate the critical phosphothreonine and phosphotyrosine residues within MAP kinases (43, 44). In this study, the rapid inactivation of MAP kinase in preheated cells was abolished by phosphatase inhibitors. Thus, heat-inducible phosphatase(s) may be involved in its rapid inactiva-
FIG. 7. HSP70 overexpression caused the early phosphorylation of MKP-1. A, MKP-1 was denatured by heat treatment. BEAS-2B cells were exposed to 43 or 37 °C for 1 h followed by incubation at 37 °C for 4 h (Pre-HS1/4). Cells were then heat-shocked for 2 h and allowed to recover for 0 or 4 h. Cells were lysed in a buffer containing 1% Triton X-100. The supernatants, which contained Triton X-100-soluble proteins, were collected after centrifugation at 2500 rpm for 5 min. Triton X-100-insoluble proteins remaining in pellets were dissolved using 2% SDS-PAGE sample buffer. B, high levels of HSP70 expression were coincident with the early appearance of p-MKP-1. Preheated BEAS-2B cells were exposed to 43 °C for 2 h and allowed to recover for 0, 1, 2, or 4 h. C, p-MKP-1 appeared earlier after HS in stably transfected cells with plasmid encoding HSP70. Stably transfected NIH3T3 cells with plasmid vector encoding HSP70 (HS0.5) or control vector (Neo) were heat-shocked for 0.5 h (HS 0.5) followed by recovery at 37 °C for 0, 4, 8, or 24 h. D, p-MKP-1 was detected earlier after HS by adenovirus-mediated HSP70 overexpression. BEAS-2B cells were infected with Ad-HSP70 or Ad-β-gal. After 24 h, cells were exposed to 43 °C for 2 h (HS2) and then allowed to recover at 37 °C for 0, 2, 4, or 5 h. E, blocking of HSP70 induction both suppressed p-MKP expression and accelerated ERK inactivation in preheated cells. BEAS-2B cells were infected with adenovirus vectors expressing HSP70 antisense RNA (Ad-AS-HSP70) or control adenovirus (Ad-β-gal). After 24 h, cells were exposed to 1 h of HS and allowed to recover for 4 h (Pre-HS1/4) and then were heat-shocked for 2 h and allowed to recover for 4 h (HS2/4). F, HSP70 associated physically with MKP-1. BEAS-2B cells were incubated at 43 °C for 1 h followed by incubation at 37 °C for 4 h (Pre-HS1/4). Cells were then heat-shocked for 2 h and allowed to recover for 4 h. Immunoprecipitates with anti-MKP-1 antibody (IP) were subjected to immunoblotting for HSP70 or MKP-1. HSP70, p-ERK, MKP-1, and actin were detected by Western blot analysis. Results are representative of three separate experiments.

The lack of either PP2A up-regulation or an increase in its activity by HS argues against the role of PP2A in the rapid inactivation of MAP kinase pathways. In contrast, HS induced MKP-1, which is consistent with previous studies (26, 45, 46). Interestingly, p-MKP-1 was detected only in preheated cells after a second HS. Previous studies have shown that the expression of MKP-1 is transcriptionally regulated by MAP kinase (26) and that MAP kinase also phosphorylates MKP-1 (29). This supports the view that preheating acts as a priming step and that the subsequent HS acts as an effecter step. Thus, increased MAP kinase activity after preheating may be responsible for the phosphorylation of MKP-1. However, in the present study, when the preheating-induced ERK activation was blocked by PD98059 pretreatment, MKP-1 phosphorylation after HS was not suppressed (data not shown), suggesting that the activation of ERK is less likely to cause MKP-1 phosphorylation in this model of experiment. MKP-1 expression was not seen following preheating in this study. This suggests that MKP-1 did not play a direct role in the priming phase and that preheating/recovery induces a priming step that is either proximal or independent of MKP-1. We demonstrated that preheating up-regulated HSP70 expression and enhanced solubility of MKP-1 as shown in Fig. 7A. Moreover, we found that MKP-1 physically associated with HSP70. Thus, considering the molecular chaperone effect of HSPs, up-regulated HSP70 during preheating/recovery seems to have a role in preventing a subsequent HS-induced MKP-1 denaturation or in facilitating renaturation after a second HS. Therefore, the priming step for the rapid inactivation of MAP kinase after a second HS is likely to be HSP70 induction.

A comparison of time courses suggested an association between the appearance of p-MKP-1 and the rapid inactivation of MAP kinase. To further confirm this, we evaluated the effect of MKP-1 up-regulation by proteasome inhibition on MAP kinase activation. In previous studies, the effects of proteasome inhibitors on the activity of MAP kinase were found to be dependent on the cells used. In U937 cells, MG132 caused JNK activation (47). In contrast, MG132 decreased p-ERK in NCI-H157 cells and in human mammary and breast cancer cells (30). Thus, the effect of proteasome inhibition on MAP kinase activity may be cell type-specific. When BEAS-2B cells were incubated with MG132 for more than 12 h, ERK and JNK activation occurred (data not shown). In order to exclude the stimulating effect of MG132 on MAP kinase activity, we tried to determine the time when MKP-1 expression was up-regulated without MAP kinase

\(^{2}\) K.-H. Lee, C.-T. Lee, Y. Whan Kim, S. Koo Han, Y.-S. Shim, and C.-G. Yoo, unpublished data.
activation. We found that p-MKP-1 expression was strongly enhanced without a change in ERK activity 6 h after MG132 treatment. Under this condition, MAP kinase inactivation after HS was accelerated. In addition, the blocking of MKP-1 induction with antisense MKP-1 oligonucleotides suppressed the rapid MAP kinase inactivation in preheated cells. These results suggest that the up-regulation of p-MKP-1 may be related to the inactivation of MAP kinase. MAP kinase was activated despite detectable levels of the unphosphorylated MKP-1 4 h after HS. In this study, we do not know whether unphosphorylated MKP-1 is active or not. However, when AS-MKP-1 was applied to cells treated with HS2/4 in which only unphosphorylated MKP-1 was induced, p-ERK expression was not augmented. In contrast, AS-MKP-1 abrogated the accelerated inactivation of ERK in preheated cells in which p-MKP-1 was present. This suggests that if unphosphorylated MKP-1 should be active, its activity may be less than that of p-MKP-1.

Neither preheating nor MG132 treatment caused cells to be refractory to HS-induced MAP kinase activation. In contrast, MG132 pretreatment completely suppressed MAP kinase activation by tumor necrosis factor α (data not shown). p-MKP-1 induced by MG132 was not affected by tumor necrosis factor α, whereas it was rapidly attenuated by denaturation due to heat stress. Thus, the inability of MKP-1 induction to make cells refractory to HS-induced MAP kinase activation may be related to the rapid disappearance of p-MKP-1 after HS, which was caused by its denaturation by HS. As molecular chaperones, HSPs prevent denaturation and assist in the renaturation of misfolded proteins (15–17). In addition, HSP70 induction was found to be associated with rapid MAP kinase inactivation by preheating. This led us to speculate as to whether HSP70 has some role in MKP-1 phosphorylation. p-MKP-1 appeared earlier in HSP70-overexpressed cells than in control cells, and blocking of HSP70 induction suppressed both p-MKP expression and accelerated ERK inactivation in preheated cells. This implies that increased HSP70 expression is required for MKP-1 phosphorylation. Thus, it appears likely that the up-regulated HSP70 after preheating may assist MKP-1 phosphorylation after a subsequent HS. Although it is not clear how HSP70 is involved in MKP-1 phosphorylation, because we found that HSP70 is associated physically with MKP-1, HSP70 in its role as a molecular chaperone might assist MKP-1 phosphorylation by preventing the denaturation of MKP-1.

To the best of our knowledge, this study is the first to find that preheating accelerates MAP kinase inactivation after a subsequent HS and that this is related to a HSP70-mediated increase in p-MKP-1. The mechanism of MKP-1 phosphorylation by HSP70 requires further detailed study.

Acknowledgments—We thank Dr. Michael Sherman (Department of Biochemistry, Boston University Medical School) and Dr. Dick Mosser (Department of Molecular Biology and Genetics, University of Guelph, Guelph, Ontario, Canada) for recombinant adenovirus vectors encoding HSP70 sense and antisense RNA.