

Activation of the Heat Shock Factor 1 by Serine Protease Inhibitors

AN EFFECT ASSOCIATED WITH NUCLEAR FACTOR- κ B INHIBITION*

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Heat shock proteins (HSPs) have a cytoprotective role in several human diseases, including ischemia and viral infection. Nuclear factor- κ B (NF- κ B) is a critical regulator of inflammation and virus replication. Here we report that a class of serine protease inhibitors with NF- κ B-inhibitory activity are potent HSP inducers via activation of heat shock transcription factor 1 (HSF1) in human cells. 3,4-Dichloroisocoumarin, the most effective compound, rapidly induces HSF1 DNA binding activity and phosphorylation, leading to transcription and translation of heat shock genes for a period of several hours. HSF1 activation is independent of *de novo* protein synthesis and is correlated in a concentration- and time-dependent manner with NF- κ B inhibition. Cysteine protease inhibitors E64 and calpain inhibitor II, which do not block NF- κ B activation, do not induce HSF1 DNA binding activity. HSP induction by 3,4-dichloroisocoumarin is associated with antiviral activity during rhabdovirus infection. These results identify a new class of HSP inducers and indicate a link between the regulatory pathways of HSF and NF- κ B, suggesting novel strategies to simultaneously switch on cytoprotective genes and down-regulate inflammatory and viral genes.

The heat shock response is a finely regulated and highly conserved mechanism to protect cells against different types of injury, including extreme temperatures, oxidative stress, and virus infection (1, 2). In mammalian cells, triggering of the heat shock response requires activation and nuclear translocation of a transregulatory protein, the heat shock transcription factor (HSF),¹ which converts from a monomeric non-DNA binding form to an oligomeric form that binds to specific promoter elements (HSEs) located upstream of heat shock (*hs*) genes and is phosphorylated by an as yet unknown kinase (2, 3). Several HSFs (HSF1–HSF4) were described in vertebrate cells (3);

however, the molecular mechanisms responsible for signal transduction, which leads to HSF activation and phosphorylation, have not been completely elucidated.

Whereas HSP induction was at first interpreted as a signal for detection of physiological stress, it is now well-documented that HSPs are utilized by the cells in the repair process following different types of injury to prevent damage resulting from the accumulation and aggregation of non-native proteins (2). Moreover, HSPs with chaperone function are expressed during normal growth conditions and are induced by biologically active molecules, including several arachidonic acid metabolites (4). We reported that cyclooxygenase cyclopentenone metabolites, such as prostaglandins of the A and J type induce the synthesis of the 70-kDa HSP (hsp70) via cycloheximide-dependent activation of HSF1 (5), and we have shown that induction of hsp70 is associated with a cytoprotective effect of prostaglandins during hyperthermia and virus infection in mammalian cells (6, 7). During these studies, we came across the interesting observation that prostaglandins, which function as signal for HSF induction, are also potent inhibitors of the activation of nuclear factor- κ B (NF- κ B) in human cells (8).

The eukaryotic transcription factor NF- κ B is a critical regulator of the immediate early pathogen response and activation of the immune system and is involved in many pathological events, including progression of AIDS (9, 10). In most cells, NF- κ B exists in an inactive cytoplasmic complex, the predominant form of which is a heterodimer composed of p50 and p65 (Rel A) subunits bound to inhibitory proteins of the I κ B family, usually I κ B α , and is activated in response to primary (*e.g.* viruses, bacteria, and UV) or secondary (*e.g.* inflammatory cytokines) pathogenic stimuli (11). Stimulation triggers rapid phosphorylation and degradation of I κ B α , resulting in NF- κ B translocation to the nucleus, where it binds to DNA at specific κ B-sites, rapidly inducing a variety of genes encoding signaling proteins. Target genes include several inflammatory and chemotactic cytokines, cytokine receptors, cell adhesion molecules, and viral genes (11), making NF- κ B an attractive therapeutic target for novel anti-inflammatory and cytoprotective drugs.

We have shown that cyclopentenone prostaglandins inhibit NF- κ B activation by preventing I κ B α phosphorylation and degradation and that this effect is tightly associated with HSF activation in human cells, suggesting that NF- κ B inhibitors might affect HSF activation (8). We now report that five distinct serine protease inhibitors with chymotrypsin-like specificity, which effectively inhibit NF- κ B by interfering with I κ B α phosphorylation and degradation (12, 13), are potent inducers of the heat shock response in human cells.

EXPERIMENTAL PROCEDURES

Cell Culture—Human Jurkat T cells, K562 erythroleukemic cells, HeLa cells, and monkey kidney epithelial MA104 cells were grown in RPMI 1640 medium (Jurkat, K562, and MA104 cells) or Dulbecco's modified Eagle's medium (HeLa cells) supplemented with 10% fetal calf

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¹ The abbreviations used are: HSF, heat shock transcription factor; HSE, heat shock element; NF- κ B, nuclear factor- κ B; TNF α , tumor necrosis factor α ; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; DCIC, 3,4-dichloroisocoumarin; TPCK, *N*- α -tosyl-L-phenylalanine chloromethyl ketone; TLCK, *N*- α -tosyl-L-lysine chloromethyl ketone; BTEE, *N*-benzoyl-L-tyrosine-ethyl ester; APNE, *N*-acetyl-DL-phenylalanine- β -naphthylester; E64, trans-epoxysuccinyl-L-leucylamido(4-guanidino)-butane; EMSA, electrophoretic mobility shift assay; MDP, Molecular Dynamics PhosphorImager; PAGE, polyacrylamide gel electrophoresis; VSV, vesicular stomatitis virus.

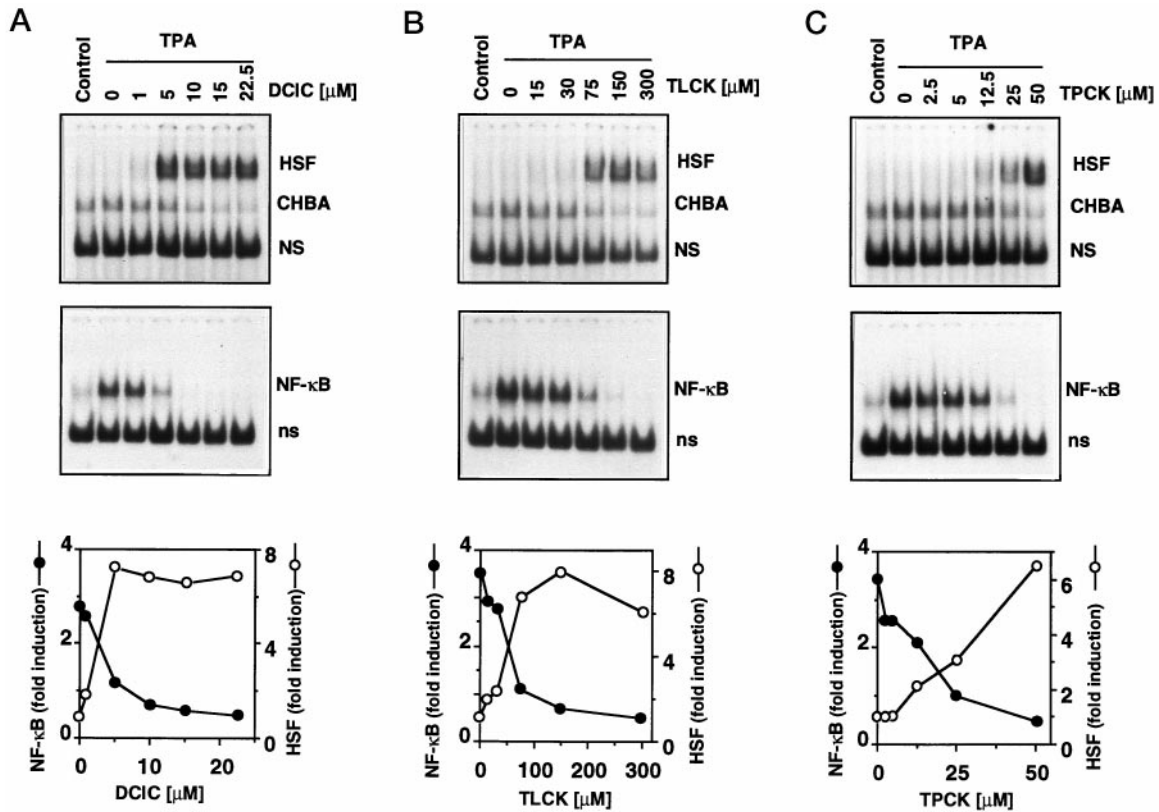


FIG. 1. Serine protease inhibitors activate HSF DNA binding activity at concentrations that inhibit NF- κ B. Jurkat cells exposed to different concentrations of DCIC (A), TLCK (B), TPCK (C), or control diluent for 1 h were stimulated with TPA (25 ng/ml). After 1 h, whole cell extracts from control or TPA-treated cells were incubated with a 32 P-labeled HSE probe (top panels) or κ B DNA probe (middle panels), followed by analysis of DNA binding activities by EMSA. Section of fluorograms from native gels are shown. Position of HSF-DNA binding complex (HSF), constitutive HSE binding activity (CHBA) (17), and nonspecific protein-DNA interactions (NS) are shown (top panels). Position of NF- κ B-DNA complex (NF- κ B) and nonspecific binding (ns) are indicated (middle panels). The levels of NF- κ B (●) and HSF (○) DNA binding activity were quantitated by MDP analysis. NF- κ B and HSF levels in arbitrary units are expressed as fold induction of NF- κ B and HSF levels in untreated unstimulated control cells (bottom panels).

serum and antibiotics at 37 °C in a 5% CO₂ atmosphere. Recombinant human tumor necrosis factor α (TNF α), 12-*O*-tetradecanoylphorbol-13-acetate (TPA), 3,4-dichloroisocoumarin (DCIC), *N*- α -tosyl-L-phenylalanine chloromethyl ketone (TPCK), *N*- α -tosyl-L-lysine chloromethyl ketone (TLCK), *N*-benzoyl-L-tyrosine-ethyl ester (BTEE), *N*-acetyl-DL-phenylalanine- β -naphthylester (APNE), and trans-epoxysuccinyl-L-leucylamido(4-guanidino)-butane (E64) were obtained from Sigma; α -N-acetyl-leu-leu-methioninal (calpain inhibitor II) was purchased from Boehringer Mannheim. TPCK was dissolved in ethanol; TLCK was dissolved in 20 mM sodium phosphate; DCIC, BTEE, APNE, E64, and calpain inhibitor II were dissolved in dimethyl sulfoxide (Me₂SO). Cells (10⁶/ml) were stimulated with 25 ng/ml TPA or 10 ng/ml TNF α in the presence of protease inhibitors or the respective control diluent. In dose-response experiments, the control received the maximal concentration of control diluent (0.1%) that in no case affected HSF or NF- κ B activation. Unless otherwise specified, cells were treated with protease inhibitors in RPMI 1640 medium containing 10% fetal calf serum starting 1 h before TPA or TNF α addition. TPA and TNF α were kept in the culture medium for the duration of the experiment. Stimulation was stopped by cooling on ice and immediate centrifugation of cells in an Eppendorf microcentrifuge.

Virus Infection and Titration—Confluent MA104 monolayers were washed with phosphate-buffered saline and infected with vesicular stomatitis virus (VSV), Indiana serotype (Orsay) (14), 1 plaque-forming unit/cell. After incubation for 1 h at 37 °C, virus inocula were removed, and monolayers were washed three times with phosphate-buffered saline and incubated with 1 ml of RPMI 1640 medium containing 2% fetal calf serum and different concentrations of DCIC. Control media contained the same concentration of Me₂SO diluent, which was shown not to affect cell or virus metabolism. For VSV titration, medium was collected 12 h postinfection, and after removing cell debris by centrifugation at 2000 rpm for 15 min, titers were determined by cytopathic effect 50% assay on confluent monolayers of MA104 cells in 96-well tissue culture dishes (six dilutions for each sample, eight wells for each

dilution) as described previously (14). The dilution that gives 50% cytopathic effect was determined by the interpolating procedure described in Reed and Muench (15).

Electrophoretic Mobility Shift Assay (EMSA)—Whole cell extracts were prepared after lysis in a high-salt extraction buffer containing 20 mM Hepes (pH 7.9), 0.35 M NaCl, 20% glycerol, 1% Nonidet P-40, 1 mM MgCl₂, 1 mM DTT, 0.5 mM EDTA, 0.1 mM EGTA, 0.2% aprotinin, 1 mM PMSF, 0.5 μ g/ml leupeptin, and 0.7 μ g/ml pepstatin (8). Whole cell extracts (15 μ g of protein) were incubated with a 32 P-labeled κ B DNA probe (16) or a HSE probe (17), followed by analysis of DNA binding activities by EMSA. Binding reactions were performed as described (17). Complexes were analyzed by nondenaturing 4% polyacrylamide gel electrophoresis. Specificity of protein-DNA complexes was verified by immunoreactivity with polyclonal antibodies specific for p65 (Rel A) or for HSF-1, for NF- κ B and HSF respectively. Quantitative evaluation of NF- κ B- κ B element and HSF-HSE complex formation was determined by Molecular Dynamics PhosphorImager (MDP) analysis. To determine the specificity of HSF1-DNA binding complexes, whole cell extracts from Jurkat cells untreated or treated with 10 μ M DCIC for 1 h were preincubated with different dilutions of anti-HSF1 or anti-HSF2 polyclonal antibodies (kindly provided by R. Morimoto, Northwestern University, Evanston, IL) for 15 min before electromobility supershift assay (14). For electromobility supershift assay of HSF-HSE binding activity in the presence of monoclonal antibodies to phosphoaminoacids, whole cell extracts (10 μ g) from Jurkat cells treated with 10 μ M DCIC for 1 h or stressed at 42 °C for 20 min were incubated with monoclonal antibodies to phosphoserine, phosphothreonine, and phosphotyrosine (clones PSR-45, PTR-8, and PT-66, respectively; Sigma) for 20 min at 21 °C prior to addition of radiolabeled HSE.

Transcriptional Run-on Assay—*In vitro* run-on transcription reactions were performed in isolated Jurkat nuclei as described (18). 32 P-Labeled RNA was hybridized with nitrocellulose filters containing plasmids for the following human genes: *hsp90* (pUCH5801) (19), *hsp70* (pH2.3) (20), *hsc70* (heat shock cognate 70, pHA7.6) (20), *grp78/BiP*

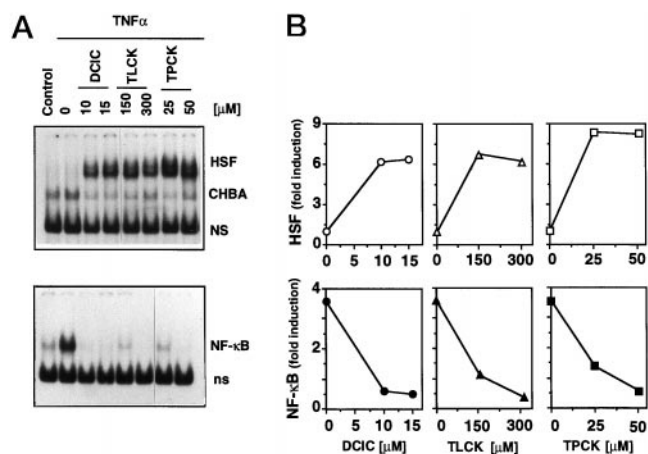


FIG. 2. Effect of serine protease inhibitors on HSF and NF- κ B activation in TNF α -stimulated Jurkat cells. Jurkat cells exposed to different concentrations of DCIC (○, ●), TLCK (△, ▲), TPCK (□, ■), or control diluent for 1 h were stimulated with TNF α (10 ng/ml). After 1 h, whole cell extracts from control or TNF α -treated cells were incubated with a 32 P-labeled HSE probe (top panel) or κ B DNA probe (bottom panel), followed by analysis of DNA binding activities by EMSA. Section of fluorograms from native gels are shown (A). Position of HSF, CHBA, and NS (top panel) are indicated as in Fig. 1. Position of NF- κ B-DNA complex (NF- κ B) and nonspecific binding (ns) are indicated (bottom panel). The levels of NF- κ B (●, ▲, ■) and HSF (○, △, □) DNA binding activity was quantitated by MDP analysis and expressed in arbitrary units as fold induction of NF- κ B and HSF levels in untreated unstimulated control cells (B).

(pHG23.1) (19), or *GAPDH* (rat glyceraldehyde phosphate dehydrogenase) (19), as a control. Following hybridization, filters were visualized by autoradiography, and the radioactivity was quantitated by MDP analysis.

Protein Synthesis and Western Blot Analysis—Jurkat or MA104 cells were labeled with L- 35 S-methionine (10 μ Ci/ 2×10^5 cells, 1-h pulses) at different times after DCIC addition (4). Cells were washed with phosphate-buffered saline and lysed in 400 μ l of lysis buffer (20 mM Tris-Cl, pH 7.4, 0.1 M NaCl, 5 mM MgCl $_2$, 1% Nonidet P-40, 0.5% SDS) containing protease inhibitors. After cell lysis, the radioactivity incorporated into TCA-insoluble material was determined, and an aliquot of samples containing the same amount of radioactivity was separated by SDS-PAGE in a vertical slab gel apparatus (3% stacking gel, 10% resolving gel), and processed for autoradiography and densitometric analysis (4). For Western blot analysis, equal amounts of protein (50 μ g/sample) from Jurkat or MA104 whole cell extracts were separated by SDS-PAGE, blotted to nitrocellulose, and filters were incubated with rabbit polyclonal anti-HSF1 or monoclonal anti-hsp70 antibodies (Amersham Pharmacia Biotech), followed by decoration with peroxidase-labeled anti-rabbit or anti-mouse IgG (ECL, Amersham Pharmacia Biotech) (8).

Statistical Analysis—Statistical analysis was performed using Student's *t* test for unpaired data. Data were expressed as the mean \pm S.E., and *P* values of < 0.05 were considered significant.

RESULTS AND DISCUSSION

Human Jurkat T cells were treated with different concentrations of the serine protease inhibitors DCIC, TPCK, and TLCK. After 1 h, cells were stimulated with the NF- κ B inducer TPA in the presence of the inhibitors. One hour after stimulation, whole cell extracts were analyzed by electrophoretic mobility shift assay to determine NF- κ B and HSF activation. The levels of NF- κ B and HSF DNA binding activity were quantitated by MDP analysis. TPA stimulated NF- κ B activation, whereas it did not induce HSF. As expected, the serine protease inhibitors prevented the induction of NF- κ B DNA binding activity in response to TPA in a dose-dependent manner (Fig. 1). DCIC was found to be the most effective compound (NF- κ B IC $_{90}$ = 5.5 μ M), followed by TPCK (IC $_{90}$ = 20 μ M) and TLCK (IC $_{90}$ = 75 μ M). Surprisingly, the serine protease inhibitors tested were found to be potent inducers of HSF activation in Jurkat cells.

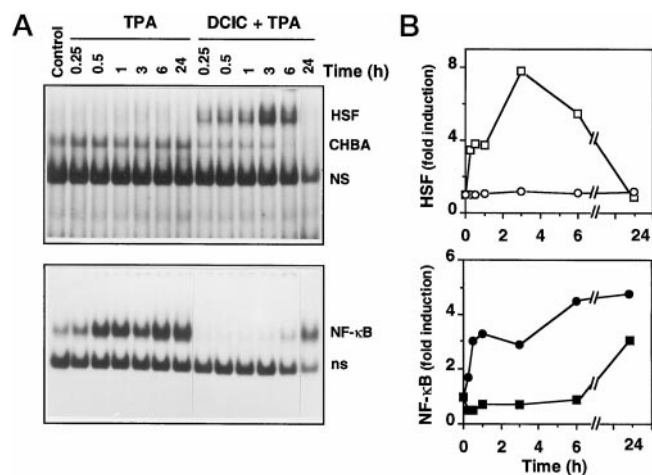


FIG. 3. Effect of DCIC on the kinetics of NF- κ B and HSF activation in TPA-stimulated cells. Jurkat cells were treated with 10 μ M DCIC (□, ■) or control diluent (○, ●) for 1 h and then stimulated with TPA. Whole cell extracts were analyzed by EMSA at different times after TPA addition. Section of fluorograms from native gels are shown (A). Positions of HSF, CHBA, and NS (top panel) are indicated as in Fig. 1. Positions of NF- κ B-DNA complex (NF- κ B) and nonspecific binding (ns) are indicated (bottom panel). B, the levels of NF- κ B (●, ■) and HSF (○, □) DNA binding activity were quantitated by MDP analysis and expressed in arbitrary units as fold induction of NF- κ B and HSF levels in untreated unstimulated control cells.

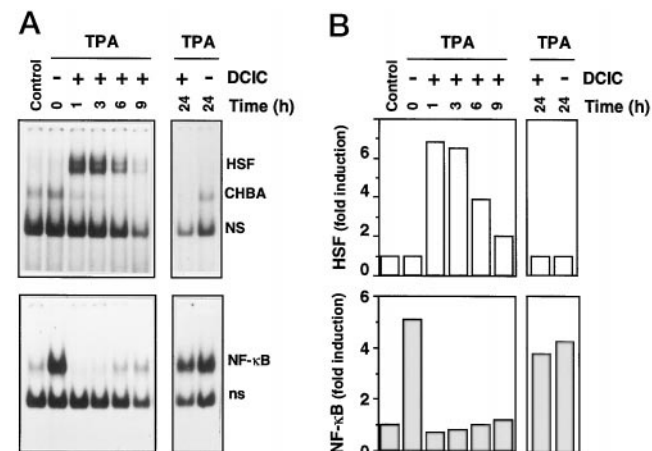


FIG. 4. Activation of HSF by DCIC is associated with NF- κ B inhibition. Jurkat cells were treated with 10 μ M DCIC for 1, 3, 6, 9, or 24 h and then stimulated with TPA. Whole cell extracts were analyzed by EMSA 30 min after TPA addition. Section of fluorograms from native gels are shown (A). Positions of HSF, CHBA, and NS (top panels) are indicated as in Fig. 1. Positions of NF- κ B-DNA complex (NF- κ B) and nonspecific binding (ns) are indicated (bottom panels). B, the levels of HSF (top panels) and NF- κ B (bottom panels) DNA binding activity were quantitated by MDP analysis and expressed in arbitrary units as fold induction of NF- κ B and HSF levels in untreated unstimulated control cells.

HSF activation was dose-dependent and generally correlated with the NF- κ B inhibitory activity of these compounds (Fig. 1). Treatment with two other serine protease inhibitors, BTEE and APNE, also prevented NF- κ B activation in TPA-treated Jurkat cells, although at higher concentrations as compared with the compounds described above (APNE, NF- κ B IC $_{90}$ = 300 μ M; BTEE, NF- κ B IC $_{90}$ = 400 μ M). Both APNE and BTEE were also found to activate HSF at concentrations that inhibited NF- κ B (data not shown). In a parallel experiment, Jurkat cells treated with different concentrations of DCIC, TPCK, TLCK, APNE, or BTEE for 1 h were stimulated with TNF α (10 ng/ml). One hour after stimulation, whole cell extracts were analyzed by EMSA, and the levels of NF- κ B and HSF DNA binding

activity were quantitated by MDP analysis. Also in this case, inhibition of NF- κ B was associated with activation of HSF in cells treated with DCIC, TPCK, and TLCK (Fig. 2), as well as with BTEE and APNE (data not shown).

To investigate whether the activation of HSF was temporally correlated with the kinetics of NF- κ B inhibition by serine protease inhibitors, Jurkat cells were treated with 10 μ M DCIC for 1 h and then stimulated with TPA. The protease inhibitor and TPA were kept in the culture medium for the length of the experiment. At different time intervals after TPA stimulation, whole cell extracts were analyzed by EMSA to determine NF- κ B and HSF activation. Under these conditions, TPA alone had no effect on HSF, whereas DCIC was found to activate HSF for a period of several hours (Fig. 3, top panels). In the absence of DCIC, NF- κ B was activated 15–30 min after TPA stimulation, and high levels of NF- κ B- κ B complexes (approximately a 5-fold increase over control) were still detected 24 h after exposure to TPA. As expected, DCIC-treatment inhibited NF- κ B activation for at least 6 h after TPA stimulation; however, after 24 h of continuous stimulation in the presence of the protease inhibitor, DCIC had lost its NF- κ B-inhibitory activity, and a 3-fold increase in the level of NF- κ B- κ B complexes was detected (Fig. 3, bottom panels). Interestingly, at this time, DCIC was no longer able to keep HSF in the activated DNA binding state (Fig. 3, top panels). In a different experiment, Jurkat cells were treated with 10 μ M DCIC for different lengths of time (1–24 h) prior to TPA stimulation. Whole cell extracts were analyzed by EMSA 30 min after TPA addition. The levels of NF- κ B and HSF DNA binding activity were quantitated by MDP analysis. As shown in Fig. 4, pretreatment with DCIC for a period of 6–9 h inhibited NF- κ B, whereas HSF was activated. Interestingly, if cells were stimulated with TPA after a 24-h continuous DCIC treatment, when HSF was no longer activated, DCIC failed to inhibit NF- κ B induction (Fig. 4, bottom panels), suggesting that the activation of HSF could be interfering with one of the signals involved in NF- κ B activation. Consistent with this suggestion, treatment of Jurkat cells with different concentrations of cysteine protease inhibitor E64 and calpain inhibitor II, which were reported not to block NF- κ B activation (24), did not induce HSF DNA binding activity in Jurkat cells (Fig. 5).

HSF activation by DCIC, the most effective NF- κ B inhibitor, was investigated in more detail. In vertebrate cells, the heat shock transcriptional response is under the control of a family of heat shock factors, which are negatively regulated. In human cells, HSF type 1 (HSF1) and HSF type 2 (HSF2), even though

they share common functional regions, are regulated differently, and whereas HSF2 is activated in differentiation or early development, HSF1 is activated under stress conditions (3). We have characterized the type of HSF activated by DCIC. As for heat shock (21, 22), HSF type 1 is the primary component of DCIC-induced HSE binding activity, as determined by electromobility supershift assay after preincubation of whole cell extracts with polyclonal anti-HSF1 or anti-HSF2 antibodies (Fig. 6A). Heat shock-induced HSF1 activation is known to be dependent on *de novo* protein synthesis in the case of a mild (42 $^{\circ}$ C) heat treatment, whereas HSF activation after severe (45 $^{\circ}$ C) heat shock is independent of cellular protein synthesis (23). In order to determine whether HSF1 activation by DCIC

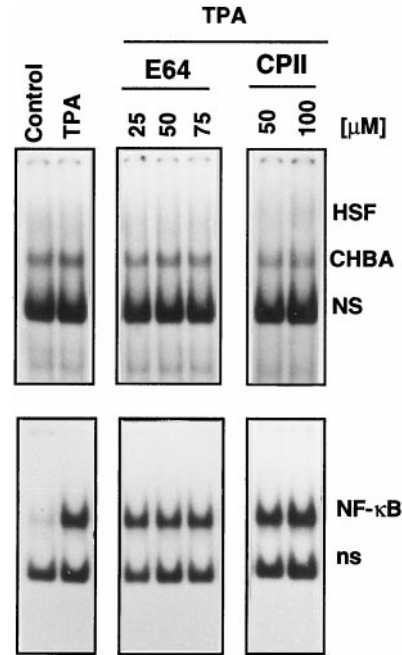


FIG. 5. Effect of protease inhibitors E64 and calpain inhibitor II (CPII) on HSF activation. Jurkat cells treated with different concentrations of E64, CPII, or Me₂SO diluent for 1 h were stimulated with TPA. After 30 min, whole cell extracts were analyzed by EMSA. Section of fluorograms from native gels are shown. Positions of HSF, CHBA, and NS (top panels) are indicated as in Fig. 1. Positions of NF- κ B-DNA complex (NF- κ B) and nonspecific binding (ns) are indicated (bottom panels).

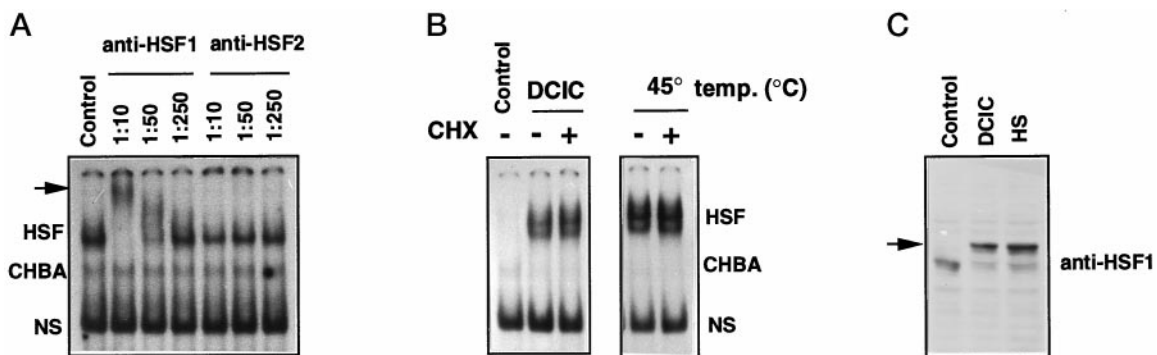


FIG. 6. DCIC-induced activation and phosphorylation of HSF1. A, specificity of HSF1-DNA binding complexes. Whole cell extracts from Jurkat cells treated with 10 μ M DCIC for 1 h were preincubated with different dilutions of anti-HSF1 or anti-HSF2 polyclonal antibodies for 15 min before electromobility supershift assay. Position of HSF, CHBA, and NS are indicated as in Fig. 1. The shifted HSF-DNA complex is indicated by the arrow. B, effect of cycloheximide on HSF activation by DCIC or heat shock. Jurkat cells kept for 30 min at 37 $^{\circ}$ C in the presence (+) or in the absence (-) of 100 μ M cycloheximide (CHX) were either treated with 10 μ M DCIC (right panel) or stressed at 45 $^{\circ}$ C for 20 min (left panel). One hour after DCIC treatment or 20 min after heat shock, whole cell extracts were prepared and subjected to EMSA. C, an equal amount of protein (50 μ g) from Jurkat cells treated with 10 μ M DCIC for 1 h or stressed at 45 $^{\circ}$ C for 20 min (HS) was separated by SDS-PAGE and processed for Western blot analysis using anti-HSF1 antibodies. A unique band of HSF1 of approximately 78 kDa was detected in unstressed Jurkat cells (Control). The higher molecular mass form of HSF1 detected in heat-shocked or DCIC-treated cells is indicated by the arrow.

was dependent on *de novo* protein synthesis, Jurkat cells were either treated with 10 μ M DCIC or were stressed at 45 °C for 20 min, in the presence or in the absence of 100 μ M cycloheximide. One hour after DCIC treatment or 20 min after heat shock, whole cell extracts were prepared and subjected to EMSA. As shown in Fig. 6B, DCIC-induced HSF1 activation was found to be independent of *de novo* protein synthesis, because it was not affected by cycloheximide.

In order for HSF1 to be transcriptionally active, apart from HSF1 trimerization, nuclear translocation, and binding to heat shock elements, inducible HSF1 phosphorylation appears to be required (22). Heat shock induces HSF1 phosphorylation (21). This modification results in the altered mobility of HSF1 on SDS-PAGE (19). We have investigated the effect of DCIC as compared with heat shock on HSF1 phosphorylation. Whole cell extracts from Jurkat cells treated with 10 μ M DCIC for 1 h or stressed at 45 °C for 20 min were processed by Western blot analysis using anti-HSF1 antibodies. A unique band of HSF1 of approximately 78 kDa was detected in unstressed Jurkat cells (Fig. 6C). As previously shown in different cell lines (21), heat shock caused a significant increase (approximately 12 kDa) in HSF1 molecular size. A similar increase in molecular size was detected in DCIC-treated cells. As previously reported for heat shock (19), the increase in HSF1 molecular mass in DCIC-treated cells was due to HSF1 phosphorylation, as shown by Western blot analysis of HSF1 after calf intestinal phosphatase digestion in the presence or the absence of the phosphatase inhibitor NaH_2PO_4 (data not shown). The fact that anti-phosphoserine monoclonal antibodies caused a change in mobility of the HSF1-HSE complex, whereas anti-threonine and anti-tyrosine antibodies did not, suggests that, similarly to heat shock (22), DCIC induced serine and not threonine or tyrosine phosphorylation.² Finally, HSF1 activation by DCIC is not specific for Jurkat cells; it is detected in HeLa and K562 human cells, as well as in MA104 monkey cells, and is obtained at concentrations that do not inhibit nucleic acid or protein synthesis (data not shown).

We have investigated the kinetics of HSF1 activation and phosphorylation by DCIC in the absence of TPA and have determined the rate of hsp mRNA transcription. Whole cell extracts prepared at different times after treatment with 10 μ M DCIC were subjected to EMSA, and the levels of HSF1 DNA binding activity in DCIC-treated cells were quantitated by MDP analysis. Induction of HSF1 DNA binding activity by DCIC was detected within 10 min after treatment (data not shown); maximal levels of HSF1/HSE complexes were detected between 1 and 6 h and decreased thereafter to go below the control level 24 h after treatment (Fig. 7, A and D). HSF1 was also phosphorylated up to 9 h after DCIC addition (Fig. 7A, bottom panel). Similar to what has been observed during prolonged heat shock (21), the decrease in HSF1 hyperphosphorylation is correlated with the attenuation of HSF1 DNA binding activity and a decrease in *hs* gene transcription (Fig. 7). In the same experiment, nuclear run-on analysis showed that DCIC induced the transcription of several *hs* genes and of the glucose-regulated *grp78/BiP* gene, with the *hsp70* gene being transcribed at the highest rate (Fig. 7, B–E). *Hsp70* mRNA transcription was detected within 1 h of DCIC addition, and transcription rates were maximal by 3–6 h and declined thereafter. High levels of *hsp70* mRNA were also detected by Northern blot analysis up to 9 h after DCIC treatment (Fig. 7, C and F). DCIC-induced *hsp70* mRNA was efficiently translated *in vivo*, and *hsp70* protein synthesis, determined by [³⁵S]-methionine incorporation followed by SDS-PAGE analysis and auto-

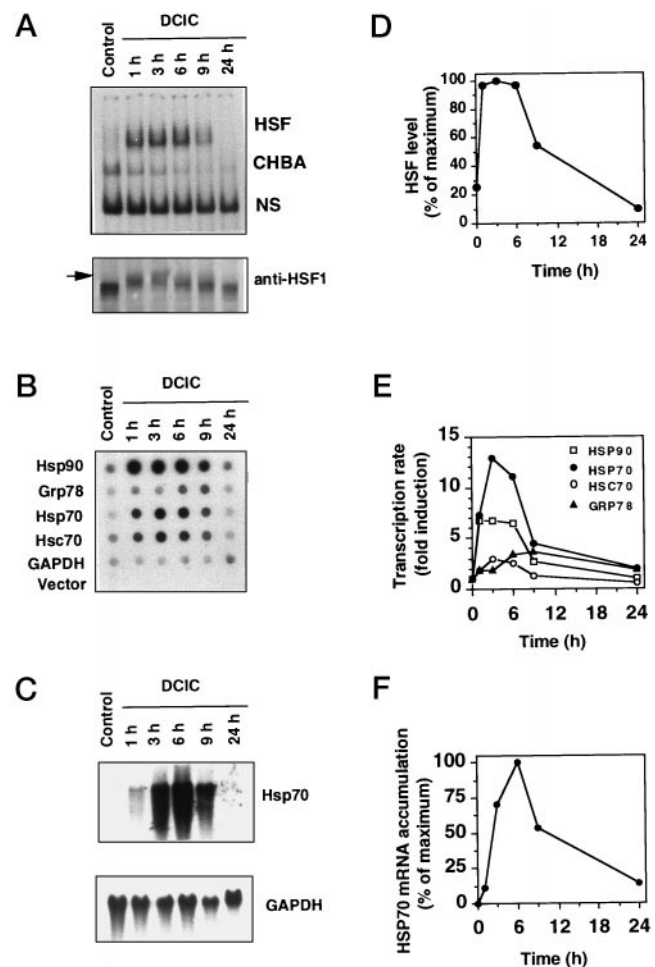


FIG. 7. Effect of DCIC treatment on HSF activation and heat shock gene transcription in Jurkat cells. A and D, kinetics of HSF activation by DCIC. Whole cell extracts prepared at different times after treatment with 10 μ M DCIC were subjected to EMSA (A, top panel). Equal amounts of protein from the same samples were separated by SDS-PAGE and assayed for HSF1 by Western blot analysis using polyclonal anti-HSF1 antibodies. A section of Western blot is shown (A, bottom panel). Arrow indicates the position of the low-mobility phosphorylated HSF1 isoform (19). Levels of HSF DNA binding activity were quantitated by MDP analysis (D). HSF values are expressed as a percentage of maximum level. B and E, transcription rates, measured by *in vivo* nuclear run-on assay. ³²P-labeled RNA was hybridized to nitrocellulose filters containing plasmids for the following human genes: *hsp90*, *grp78/BiP*, *hsp70*, *hsc70*, or GAPDH. Following hybridization, filters were visualized by autoradiography (right panel), and the radioactivity was quantitated by MDP analysis (E). The values are expressed as arbitrary units obtained by comparing transcription rates to control levels. C and F, in the same experiment, cytoplasmic RNA was subjected to Northern blot analysis using ³²P-labeled pH 2.3 (*hsp70*) or GAPDH probes. The radioactivity was quantitated by MDP analysis (F). Values normalized relative to GAPDH are expressed as a percentage of maximum level.

radiography, was sustained for at least 9 h after treatment (data not shown).

HSPs have been shown to have cytoprotective activity in a variety of human diseases, including ischemia, inflammation, and infection *in vitro* and *in vivo* (2). In cardiac tissues, HSPs were shown to play a pivotal role in restoring normal cardiac function after injury, possibly by removal of denatured cardiac proteins and re-establishment of normal cardiac protein synthesis (25, 26). HSPs were also shown to protect mammalian cells by tumor necrosis factors α - and β -mediated cytotoxicity (27), as well as to suppress astroglial-inducible nitric-oxide synthase expression (28), and to prevent lethality in a rodent model of adult respiratory distress syndrome (29). Elevated

² A. Rossi and G. Santoro, unpublished observation.

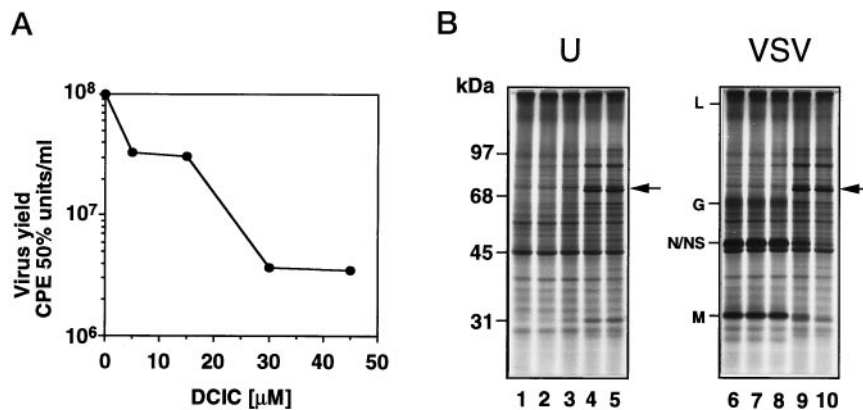


FIG. 8. Effect of DCIC on VSV replication and protein synthesis. A, confluent monolayers of MA104 cells infected with VSV were treated with different concentrations of DCIC or control diluent, starting 1 h after the adsorption period. VSV titers were determined 12 h postinfection. B, uninfected (U) or VSV-infected (VSV) MA104 cells were treated with 10 (lanes 2 and 7), 20 (lanes 3 and 8), 30 (lanes 4 and 9), 50 (lanes 5 and 10) μM DCIC or with control diluent (lanes 1 and 6) soon after VSV infection and labeled with [³⁵S]methionine (1 h pulse starting 5 h postinfection). Equal amounts of protein were analyzed by SDS-PAGE gels and autoradiography. The position of hsp70, identified by Western blot analysis (data not shown), is indicated by the arrow. VSV proteins L, G, N, NS, and M (14) are indicated.

intracellular hsp70 levels after treatment with a variety of HSP inducers, including antiviral prostaglandins, sodium arsenite, azetidine, 2-cyclopenten-1-one, and heat shock itself, were also associated with cytoprotection and inhibition of viral protein synthesis during infection with negative-strand RNA viruses (reviewed in Refs. 7 and 30). To investigate whether HSP induction by DCIC was biologically relevant and could result in a cytoprotective effect during viral infection, epithelial monkey kidney MA104 cells were infected with VSV, and immediately after the 1-h adsorption period, cells were treated with DCIC at concentrations that induced hsp70 synthesis. VSV titers were determined 12 h postinfection by cytopathic effect 50% assay. As shown in Fig. 8A, DCIC was found to inhibit the production of VSV infectious particles in a dose-dependent manner. To determine the effect of DCIC on hsp70 induction and virus protein synthesis, uninfected or VSV-infected MA104 cells were treated with different concentrations of DCIC or with control diluent as described above and labeled with [³⁵S]methionine (8 μCi/2 × 10⁵ cells, 1-h pulse starting 5 h after infection). Equal amounts of protein were analyzed on 10% SDS-PAGE gels and processed for autoradiography. Viral proteins L, G, N, NS, and M were detected in untreated VSV-infected cells at this time (Fig. 8B, lane 6). As previously shown for other hsp70 inducers (7, 14, 31), DCIC treatment selectively inhibited the synthesis of viral proteins L, G, N, NS, and M at concentrations of 30 and 50 μM (Fig. 8B, lanes 9 and 10, respectively). This effect was associated with hsp70 induction by DCIC (Fig. 8B, arrows). Inhibition of virus replication was reversible, decreasing 24 h after DCIC addition in concomitance with decreased hsp70 transcription and translation (data not shown).

The cytoprotective role of HSPs in several human diseases makes HSP inducers an attractive target for therapeutic intervention. We have now identified serine protease inhibitors as a new class of HSP inducers, acting via HSF1 stimulation. The mechanism by which these molecules activate HSF1 remains to be elucidated. DCIC inhibits serine protease by covalently binding to the catalytic serine and/or histidine residue (32). TPCK and TLCK are hydrophobic low molecular weight molecules, which penetrate the cell membrane and act intracellularly by reacting irreversibly with the histidine residue in the active center of proteases, with a high affinity for chymotrypsin and chymotrypsin-like enzymes (33). However, an involvement of serine proteases in HSF activation has never been described. HSF1 itself is not a labile protein (21), and short-lived regulators of HSF are not known. Both TLCK and TPCK can co-

valently modify -SH groups of proteins, altering protein conformation and mobility (34). DCIC has also been shown to directly modify and inhibit the activity of nonproteolytic proteins (35, 36). Because protein modification is a well-recognized signal for HSF activation (3), it could be argued that these molecules act by provoking protein alterations either directly or by inhibiting protease function. In particular, inhibition of chymotrypsin-like proteases could cause a rise in the levels of hydrophobic peptides, which would be expected to be powerful inducers of the heat shock response (37). The accumulation of abnormal proteins and/or the inhibition of the degradation of a key regulatory factor were hypothesized to be responsible for increased hsp and ER chaperone mRNA levels in canine kidney cells treated with a variety of proteasome inhibitors, including the peptide aldehyde MG132 (38). We have recently found that MG132 is also a potent activator of HSF1 in Jurkat cells.² However, it should be pointed out that other protease inhibitors, including cysteine protease inhibitors and calpain inhibitors, did not activate HSF1. The possibility that HSF1 activation by DCIC could be triggered by blocking the function of a specific serine protease or could be the result of direct binding of DCIC to selected target proteins, including HSF1 itself, cannot be ruled out.

Serine protease inhibitors have been widely used as a tool to investigate the role of NF-κB in inflammation and virus infection (13, 39, 40). Our results indicate that these and future studies have to be evaluated in a new context, in which the effect of the activation of the heat shock response by these molecules is considered.

Also, the finding that NF-κB inhibition by serine protease inhibitors, as well as by several other HSF inducers (8), is strictly associated with HSF1 activation suggests a link between the regulatory pathways of these two important transcription factors. Unlike other protease inhibitors, the serine protease inhibitors that we examined block not only the proteasome-dependent degradation of IκBα but also its phosphorylation (36, 41), whereas they induce HSF1 phosphorylation, suggesting the possibility of a controlling mechanism involving kinase modulation. Triggering of HSF1 activation could render cells unresponsive to stimulation of NF-κB. This hypothesis is very attractive, considering the opposite roles of HSF and NF-κB in cytoprotection and cell injury, respectively, as in the case of inflammation (27, 28) and viral infection (29, 42), and suggests novel therapeutic strategies relying upon simultaneous switching on of cytoprotective genes and down-regulation of inflammatory and viral genes.

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