Metabolic Oxidative Stress-induced HSP70 Gene Expression Is Mediated through SAPK Pathway

ROLE OF Bcl-2 AND c-Jun NH$_2$-TERMINAL KINASE*

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In previous reports we demonstrated that glucose deprivation induces metabolic oxidative stress in drug-resistant human breast carcinoma MCF-7/ADR cells (Lee, Y. J., Galoforo, S. S., Berns, c. M., Chen, J. C., Davis, B. H., Swim, J. E., Corry, P. M., and Spitz, D. R. (1998) J. Biol. Chem. 273, 5294–5299). In the study described here, we investigated intracellular responses to metabolic oxidative stress. Northern blots show an increase in the levels of HSP70 and HSP28 mRNAs in cells exposed to glucose-free medium for 1 h. One- and two-dimensional polyacrylamide gel analyses confirmed that glucose deprivation induced a family of HSPs, particularly an inducible HSP70. Overexpression of bcl-2 suppressed glucose deprivation-induced HSP70 gene expression, heat shock transcription factor heat shock element binding activity, as well as c-Jun NH$_2$-terminal kinase (JNK1) activation. Expression of a dominant-negative mutant of JNK1 also suppressed glucose deprivation-induced JNK1 activation as well as HSP70 gene expression. Taken together, the stress-activated protein kinase signal transduction pathway is involved in glucose deprivation-induced heat shock gene expression.

It is well established that the transcriptional induction of heat shock genes in eukaryotes is mediated by the heat shock transcription factor (Hsf) (1–8). This protein can be activated by a variety of stresses such as heat shock, heavy metals, or arsenite (4, 9–11). The activated Hsf binds to the promoters which contain the heat shock element (HSE) and then stimulates transcription (1, 5, 12). A fundamental question which remains unanswered is how these stresses activate Hsf. Hsf is phosphorylated under normal growth conditions and is hyperphosphorylated subsequent to stress. It has been proposed that such phosphorylation may be mediated through the MAP kinase family such as c-Jun NH$_2$-terminal kinase (JNK) (13). Several reports have demonstrated that the JNK, also referred to as stress-activated protein kinase (SAPK), signal transduction pathway can be activated by oxidative stress (14–16). Following activation, JNK phosphorylates several transcription factors including activating transcription factor-2 (17), Elk-1 (18), Sap-1a (19), and c-Jun (20) which are involved in regulating numerous genes implicated in cell proliferation, transformation, differentiation, and DNA repair (21–24). Recent studies also show that oxidative stress can activate HSF and increase HSP70 gene expression (25). These observations suggest a possibility that oxidative stress-induced activation of HSF is mediated through the SAPK pathway.

We have previously observed that glucose deprivation increases intracellular hydroperoxide production and oxidized glutathione in drug-resistant human breast carcinoma MCF-7/ADR cells (26). These results led us to investigate the possibility that glucose deprivation-induced metabolic oxidative stress may activate HSF through the SAPK pathway and result in hsp gene expression. In this study, we demonstrate that glucose deprivation induces HSP genes, in particular inducible HSP70 gene expression as well as JNK activation. Studies with the bcl-2 gene or dominant-negative JNK1 mutant transfected cells indicate that metabolic oxidative stress-induced HSP70 gene expression is mediated through the SAPK pathway.

MATERIALS AND METHODS

Cell Culture and Survival Determination—Multidrug-resistant human breast carcinoma (MCF-7/ADR) cells were cultured in McCoy’s 5a medium with 10% iron-supplemented bovine calf serum (HyClone, Logan, UT) and 26 mM sodium bicarbonate for monolayer cell culture. Two or three days prior to the experiment, cells were plated into 60-mm Petri dishes or T-25 flasks. The Petri dishes/flasks containing cells were kept in a 37 °C humidified incubator with a mixture of 95% air and 5% CO$_2$. For survival determination, the T-25 flasks containing monolayers of asynchronous cells were trypsinized with ice-cold 0.05% trypsin in balanced salt solution and 0.53 mM EDTA. After trypsinization, the cells were resuspended in 3 ml of McCoy’s 5a medium containing 10% iron-supplemented bovine calf serum. Cell counts were determined with a Coulter counter, and appropriate dilutions were made.

Glucose Deprivation—Cells were rinsed three times with Hanks’ balanced salt solution which took approximately 10 min. Cells were then treated with glucose-free McCoy’s 5a medium (Life Technologies, Inc., Gaithersburg, MD).

Hyperthermic Treatment—Monolayer cells were heated in a hot water bath as described previously (27).

Transfection—Exponentially growing cells were plated 2 days before experiments at a concentration of 4 x 10$^5$ cells/60-mm culture dish. Cells were transfected with pcDNA3-FLAG-JNK1 (APF) or pCMV-bcl-2 vector (20 μg) by using Lipofectin™ Reagent (Life Technologies, Inc.). The pcDNA3-FLAG-JNK1 (APF) vector contains a dominant-negative mutant of JNK1 with a NH$_2$-terminal FLAG tag, which was
kindly provided by Dr. R. Davis (Howard Hughes Medical Institute Research Laboratories, University of Massachusetts Medical Center, Worcester, MA). The pCMV-bcl-2 vector containing the bcl-2 gene was provided by Dr. S. J. Korsmeyer (Washington University, St. Louis, MO).

Labeling, One-or Two-dimensional Polyacrylamide Gel Electrophoresis (PAGE), and Fluorography—Cells were labeled with 20 μCi/ml [3H]leucine (specific activity 160 Ci/mmol, Amersham) in leucine-free medium. After labeling, cells were washed twice with cold Hanks’ balanced salt solution. For one-dimensional PAGE, samples were mixed with 2 × Laemmli lysis buffer (1 × = 2.4 μg/mL glycerol, 0.14 μL/mL Tris, pH 6.8, 0.21 μL/mL SDS, 0.01 μL/mL bromphenol blue), and boiled for 10 min. Protein content was measured using BCA® Protein Assay Reagent (Pierce, Rockford, IL). The samples were diluted with 1 × lysis buffer containing 1.28 μL/mL mercaptoethanol and an equal amount of protein (30 μg) was applied to a one-dimensional PAGE. Electrophoresis was carried out on a 10–18% linear gradient SDS-PAGE. For two-dimensional PAGE, samples were solubilized in sample buffer containing 8 M urea, 1.7% Nonidet P-40, and 4.3% β-mercaptoethanol. Proteins were first separated in isoelectric focusing gels (pH 3.5–10). These gels were then laid across the top of a 10–18% linear gradient SDS-polyacrylamide gels for two-dimensional analysis (28). After electrophoresis, gels were fixed in 30% trichloroacetic acid for 30 min. For fluorography, gels were dehydrated by washing for 15 min in each of 25% acetic acid, 50% acetic acid, and 25% isopropanol acid, consecutively. After fixation, gels were placed in 125 ml of PPO solution (20% [w/v] 2,5-diphenyloxazole in glacial acetic acid) for 2 h. The PPO solution was removed, and the gel was shaken gently overnight in distilled water and dried for 2.5 h at 60 °C. The gel was loaded into a cassette with Kodak SB-5 x-ray film and placed in a −70 °C freezer. After optimum exposure time, the fluorograph film was developed with Kodak GBX developer and fixed with Kodak GBX fixer.

Western Blot—After electrophoresis, the proteins were transferred onto a nitrocellulose membrane by electroblotting using 0.12 A and 300 V overnight. The membrane was then incubated in blocking solution (3% bovine serum albumin for non-ECL system or 5% dry milk for ECL system) for 1 h, washed, and then incubated with anti-bcl-2 monoclonal antibody (diluted 1:1,000 for ECL system; Eastman Kodak Co., New Haven, CT), anti-ACTIVETM JNK polyclonal antibody (diluted 1:5,000 in 3% bovine serum albumin for non-ECL system or 5% dry milk for ECL system; Promega, Madison, WI) or anti-actin monoclonal antibody (diluted 1:10,000; ICN, Costa Mesa, CA). After incubation with the primary antibody, the membrane was washed, and incubated with alkaline phosphatase-conjugated rabbit-anti-mouse IgG (diluted 2,000; Zymed Laboratories Inc., South San Francisco, CA) for 1–2 h or biotinylated sheep anti-mouse IgGlodenoxy anti-rabbit IgG (diluted 1:2, 5000; Amersham, Arlington Heights, IL) for 1–2 h. The membrane was then stained using either nitro blue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate or the detection reagent of the ECL detection kit (Amersham).

Northern Blot Analysis—HSP70 and HSP28 mRNA levels were determined using the Northern blot technique. Total cellular RNA was extracted using the LiCl-urea method of Tsujihara et al. (29). For RNA analysis, 30 μg of total RNA was electrophoresed in a 1% agarose-formaldehyde gel (30). The RNA was blotted from the gels onto nitrocellulose membranes and baked at 80 °C for 2 h in a vacuum oven. Membranes were prehybridized at 42 °C in 50% formamide, 1 × Denhardt’s solution, 25 mKPO4 (pH 7.4), 5 × SSC (1 × SSC = 150 mM NaCl, 15 mM sodium citrate), and 50 μg/mL salmon sperm DNA. Hybridizations were at 42 °C in prehybridization solution containing 2.4 M glycerol, 0.14 M Tris, pH 6.8, 0.21 M SDS, 2 mM dithiothreitol for HSE) containing about 0.5 ng of radioactive labeled probe for HSP70 or HSP28 mRNA. The membrane was then incubated in blocking solution (1% bovine serum albumin for non-ECL system or 5% dry milk for ECL system; Promega, Madison, WI) or anti-actin monoclonal antibody (diluted 1:10,000; ICN, Costa Mesa, CA). After incubation with the primary antibody, the membrane was washed, and incubated with alkaline phosphatase-conjugated rabbit-anti-mouse IgG (diluted 2,000; Zymed Laboratories Inc., South San Francisco, CA) for 1–2 h or biotinylated sheep anti-mouse IgGlodenoxy anti-rabbit IgG (diluted 1:2, 5000; Amersham, Arlington Heights, IL) for 1–2 h. The membrane was then stained using either nitro blue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate or the detection reagent of the ECL detection kit (Amersham).

RESULTS

Glucose Deprivation-induced HSP70 and HSP28 Gene Expression—To investigate whether glucose deprivation can activate heat shock gene expression, MCF-7/ADR cells were exposed to glucose-free medium for 1 h and then incubated in complete medium for the times (0–8 h) indicated at the bottom of each lane. Cells were harvested and RNA was isolated. An equal amount of RNA (30 μg) was loaded onto each lane of an agarose-formaldehyde gel for separation. After separation, RNA was blotted onto a nitrocellulose membrane and hybridized with a 32P-labeled probe for HSP70 or HSP28 mRNA. The membrane was then incubated in blocking solution (3% bovine serum albumin for non-ECL system or 5% dry milk for ECL system) for 1 h, washed, and then incubated with anti-bcl-2 monoclonal antibody (diluted 1:1,000 for ECL system; Eastman Kodak Co., New Haven, CT), anti-ACTIVETM JNK polyclonal antibody (diluted 1:5,000 in 3% bovine serum albumin for non-ECL system or 5% dry milk for ECL system; Promega, Madison, WI) or anti-actin monoclonal antibody (diluted 1:10,000; ICN, Costa Mesa, CA). After incubation with the primary antibody, the membrane was washed, and incubated with alkaline phosphatase-conjugated rabbit-anti-mouse IgG (diluted 2,000; Zymed Laboratories Inc., South San Francisco, CA) for 1–2 h or biotinylated sheep anti-mouse IgG (diluted 1:2, 5000; Amersham, Arlington Heights, IL) for 1–2 h. The membrane was then stained using either nitro blue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate or the detection reagent of the ECL detection kit (Amersham).

Quantitation of HSF-HSE Binding Activity—Conditions for the gel mobility shift assay, a description of the 32P-labeled HSE oligonucleotide, and preparation of whole cell extracts were as published previously (31–33). A double-stranded HSE (upper strand 5′-CTTACAGCAAAAGTTACGAGATGGGAGCTGA-3′, Ref. 34) oligonucleotide of the highly conserved HSP70 gene promoter was used. Biotin nucleotides represent essential sites for HSF. Binding reactions with 20 μg of whole cell extracts for HSE were performed for 15 min at 25 °C in a final volume of 25 μl of binding buffer (10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 1 mM EDTA, 5% glycerol, 1 mM dithiothreitol for HSF, 10 mM HEPES, pH 8.0, 15% glycerol, 2 mM EDTA, 0.5 mM spermine, 20 mM NaCl, 4 mM MgCl2, 2 mM dithiothreitol for HSF) containing about 0.5 μg of radio-
level of inducible HSP70 but not constitutive HSP70 (Fig. 3C). In contrast, the level of both HSP70s increased after heat shock (Fig. 3B). These two forms of HSP70 which can be separated by their molecular size and charge are highly related to each other. The constitutive HSP70, 73 kDa, is an abundant protein in the normal unstressed cell. The inducible HSP70, 72 kDa, is synthesized at very high levels after stress. In most cells, synthesis of the inducible HSP70 occurs only after stress. However, in human cells, synthesis of both constitutive and inducible HSP70 is observed in cells grown under normal conditions (Fig. 3A).

The Time Course for Development of Resistance to Glucose Deprivation-induced Cytotoxicity and Heat Shock—It is well known that heat shock proteins are involved in the development of tolerance to stresses (35). To examine whether glucose deprivation-induced heat shock proteins can enhance resistance to stresses, MCF-7/ADR cells were exposed to glucose-free medium for 1 h and then incubated for various times in complete medium before being challenged to glucose deprivation for 4 h (Fig. 4) or heat shock at 45 °C for 1 h (Fig. 5). Survival fraction was plotted as a function of various times (2–16 h) between pretreatment and challenge treatment. Data from Figs. 4 and 5 show that tolerance rapidly developed within 5 h and was maintained over 16 h. The time course for development of tolerance was similar for the two types of stress. For example, tolerance to glucose deprivation-induced cytotoxicity was observed as a 15-fold increase in survival from $4 \times 10^{-2}$ to $6 \times 10^{-1}$ after 8 h in complete medium (Fig. 4). In parallel, thermostolerance was observed as a 17-fold increase in survival from $4 \times 10^{-2}$ to $7 \times 10^{-1}$ after 8 h in complete medium (Fig. 5).

Role of Bcl-2 in Glucose Deprivation-induced Heat Shock Gene Expression—Previous studies have shown that glucose deprivation induces an increase in intracellular hydroperoxide production (26) which may lead to activation of heat shock gene expression. We have also observed that overexpression of bcl-2 prevents an increase in oxidized glutathione content which is an indicator of oxidative stress (data not shown). In this study, we investigated whether overexpression of bcl-2 prevents glucose deprivation-induced heat shock gene expression. For this study, cells were transfected with plasmid pCMV-bcl-2 containing the human BCL-2 cDNA gene and then stable transfecants were selected. Fig. 6 shows an overexpression of bcl-2 in pCMV-bcl-2 (pBcl-2) vector transfected cells. The level of bcl-2 did not significantly change during incubation in complete medium after 1 h of glucose deprivation in control vector pCMV (pNeo) or pbcl-2-transfected cells. Effects of overexpression of bcl-2 on glucose deprivation-induced synthesis of heat shock proteins was observed in these transfected cells (Fig. 7). Data from two-dimensional SDS-PAGE show that glucose deprivation-induced inducible HSP70 gene expression was markedly reduced in pBcl-2 transfected cells (Fig. 7). To determine whether the suppression of HSP70 gene expression was due to the alteration of upstream regulation of transcription, HSF-HSE binding activity was measured by gel mobility shift assay. It is known that the binding of HSF to HSE is necessary for transcriptional activation of eukaryotic heat shock genes (5, 36). Gel mobility shift analysis of whole cell extracts from glucose-

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**Fig. 2.** One-dimensional SDS-polyacrylamide gel electrophoretic analysis of proteins. MCF-7/ADR cells were exposed to glucose-free medium for 1 h and then labeled with $20 \mu$Ci/ml [3H]leucine in complete medium for 2–8 h, as indicated at the bottom of each lane. Lysates from cells were analyzed and 3H-labeled proteins were detected by fluorography. The location of inducible HSP70 is identified. C, untreated control cells.

**Fig. 3.** Two-dimensional SDS-polyacrylamide gel electrophoretic analysis of proteins. MCF-7/ADR cells were heated at 45 °C for 15 min (panel B) or exposed to glucose-free medium for 1 h (panel C) and then labeled with $20 \mu$Ci/ml [3H]leucine for 6 h in glucose-free medium. Lysates from cells were analyzed and 3H-labeled proteins were detected by fluorography. Only a section of the fluorograph is shown. The locations of HSP28a (a), HSP28b (b), constitutive HSP70 (c), inducible HSP70 (b), HSP90, HSP110, and actin (A) are identified. Panel A, unheated control cells.
Metabolic Oxidative Stress-induced Gene Expression

Role of SAPK Pathway in Glucose Deprivation-induced Heat Shock Gene Expression—To further evaluate the involvement of the signal transduction pathway in glucose deprivation-induced heat shock gene expression, cells were transfected with the plasmid pcDNA3-FLAG-JNK1 (APF) containing the dominant-negative mutant of JNK1 which blocks the SAPK pathway (17). Stable transfectants were exposed to glucose-free medium for various times (5–120 min) and the active form of JNK1 was detected by immunoblot assay (Fig. 10) or HSP70 synthesis was determined by one-dimensional SDS-PAGE analysis (Fig. 11). Figs. 10 and 11 show that glucose deprivation-induced JNK1 activation as well as HSP70 synthesis was markedly reduced in pcDNA3-FLAG-JNK1 (APF)-transfected cells.

DISCUSSION

Several conclusions can be drawn upon consideration of the data presented. Glucose deprivation which is known to induce metabolic oxidative stress activates HSF and subsequently induces heat shock gene expression, in particular inducible HSP70 gene expression (Figs. 1–3 and 8). These results are consistent with observations which demonstrate that oxidative stress can activate HSF and induce HSP70 gene expression (25). These observations were confirmed by overexpression of bcl-2 which may regulate an antioxidant pathway at sites of free radical generation (38, 39). Our preliminary studies of measuring pro-oxidant production by using an oxidation-sensitive fluorescent probe, 5,6-carboxy-2′,7′-dichlorofluorescein diacetate (C-400) demonstrate no capacity for bcl-2 to block peroxide generation per se during glucose deprivation (data not shown). However, bcl-2 blocks peroxide-induced damaging effects. These observations are consistent with results from oximetry measurements and menadione treatment (39). At this time, we can only speculate on the mechanism of bcl-2-induced protective effects. The majority of bcl-2 which is known to be localized as an integral mitochondrial protein may trap hydroperoxide molecules inside the mitochondria and prevent leakage. Obviously, further studies are necessary to understand the biochemical function of bcl-2. Data from Figs. 7 and 8 demonstrate that overexpression of bcl-2 suppressed glucose deprivation-induced HSF activation as well as HSP70 gene expression. Prevention of the metabolic oxidative stress-induced SAPK pathway resulted in this suppression (Fig. 9) strongly suggesting that metabolic oxidative stress induces the SAPK pathway (13, 37) and activation of HSF and HSP70 gene expression is mediated through the SAPK pathway (13). The involvement of the SAPK pathway in HSP70 gene expression was confirmed by overexpression of the dominant-negative mutant of JNK1 (Figs. 10 and 11).

We previously reported that glucose deprivation increases the steady state levels of intracellular pro-oxidants (26). An alteration in the intracellular oxidative/reduction equilibrium state may cause the activation of Src family proteins, in particular Lyn kinase which may be responsible for activation of JNK1 (40). Although the source of the increased pro-oxidant production caused by glucose deprivation is uncertain, several studies support the hypothesis that intracellular hydroperoxide production by mitochondrial metabolism may be involved. We previously demonstrated a significant increase in the steady state levels of oxidized glutathione (GSSG) during glucose deprivation (26). Wilhelm et al. (41) reported that the intracellular reduced glutathione (GSH) level was critical for JNK activation: enhancing the GSH level by pretreatment of the cell with GSH or N-acetylcysteine inhibited JNK activation, whereas depletion of the cellular GSH pool caused hyperinduction of JNK activity (41). In support of these observations, overexpression of bcl-2 prevented an increase in oxidized glutathione content (data not shown) and suppressed the activa-

deprived cells showed the formation of the HSF-HSE complex (Fig. 8). The HSF binding activity was markedly reduced in pBcl-2 transfected cells. This may be due to inhibition of the signal transduction pathway by reducing oxidative damage in pBcl-2-transfected cells. It is well known that metabolic oxidative stress induces the SAPK pathway (37) which has been suggested to be involved in the activation of HSF (13). Western blots in Fig. 9 show that glucose deprivation indeed activated JNK1 and this activation was markedly reduced in pBcl-2-transfected cells.

Fig. 4. The time course for development of resistance to glucose deprivation-induced cytotoxicity. ■, cells were incubated for various intervals (2–16 h) in complete medium after pretreatment with glucose-free medium for 1 h and then challenged to heat shock at 45 °C for 1 h. □, cells were incubated for glucose-free medium for 4 h without pretreatment. The survival fraction was plotted as a function of various incubation intervals in complete medium. The data are a compilation of two separate experiments.

Fig. 5. The time course for thermotolerance development by glucose deprivation. ■, cells were incubated for various intervals (2–16 h) in complete medium after pretreatment with glucose-free medium for 1 h and then challenged to heat shock at 45 °C for 1 h. □, cells were heated at 45 °C for 1 h without pretreatment. The survival fraction was plotted as a function of various incubation intervals in complete medium. The data are a compilation of two separate experiments.

Fig. 6. Western blots with an anti-bcl-2 antibody. MCF-7/ADR cells stably transfected with control plasmid pCMV (pNeo) or pCMV-bcl-2 (pBcl-2) were exposed to glucose-free medium for 1 h and then incubated for various intervals (0–8 h) as indicated at the bottom of each lane. Cells were harvested and 30 μg of protein from each lysate was resolved on SDS-PAGE and immunoblotted.
tion of JNK1 during glucose deprivation (Fig. 9). A fundamental question which remains unanswered is how metabolic oxidative stress triggers the SAPK signaling pathway. At this time, we can only speculate that the presence of redox-sensing molecules which can recognize the intracellular oxidative/reduction equilibrium state may be involved in the initiation of the signal transduction pathway in mammalian cells. Unlike mammalian cells, redox-sensing molecules have been well studied in bacteria.

SoxR and OxyR are redox status sensors and transcription factors which activate antioxidant genes in response to superoxide anion and H$_2$O$_2$ in *E. coli* (42). SoxR is activated by superoxide anion generating agents or by nitric oxide through two consecutive steps of gene activation (43, 44). Two forms of SoxR have been characterized: Fe-SoxR contains non-heme iron and apo-SoxR is devoid of iron or other metals (45). Both forms of SoxR bind to the *in vivo* target, the soxS promoter. However, only Fe-SoxR stimulated transcription initiation at soxS (46). The Fe-SoxR is a homodimer, and each monomer has a redox-active [2Fe-2S] cluster. The oxidation state of [2Fe-2S] centers controls its activity as a transcription activator independent of DNA binding ability (47, 48).

FIG. 7. Two-dimensional SDS-polyacrylamide gel electrophoretic analysis of proteins in pCMV (pNeo) or pCMV-bcl-2 transfected (pBcl-2) MCF-7/ADR cells. Panels B and D, cells were exposed to glucose-free medium for 1 h and then labeled with 20 μCi/ml [3H]leucine for 6 h in leucine-free medium. Lysates from cells were analyzed and 3H-labeled proteins were detected by fluorography. Only a section of the fluorograph is shown. The locations of constitutive HSP70 (a), inducible HSP70 (b), and actin (A) are identified. Panels A and C, untreated control cells.

FIG. 8. Detection of a HSF-HSE in control plasmid pCMV (pNeo) or pCMV-bcl-2 (pBcl-2) transfected MCF-7/ADR cells. Cells were exposed to glucose-free medium for 1 h and then incubated in complete medium for various intervals (0–2 h). The gel mobility shift assay was performed with a $^{32}$P-labeled HSE and whole cell extracts (20 μg of protein) prepared from untreated control cells (C) or treated cells (0–2). Closed arrow indicates the position of the HSF-HSE complex. Open arrow indicates a free $^{32}$P-labeled oligonucleotide fragment.

FIG. 9. Immunoblot detection of active form of JNK1 or actin in transfected MCF-7/ADR cells. Cells were stably transfected with pNeo or pBcl-2. Transfectants were exposed to glucose-free medium for various intervals (5–120 min) as indicated at the bottom of each lane. Western blot analysis was done as described in the legend to Fig. 6 with anti-ACTIVE JNK antibody or anti-actin antibody. C, untreated control cells.

FIG. 10. Immunoblot detection of active form of JNK1 or actin in transfected MCF-7/ADR cells. Cells were stably transfected with pNeo or dominant-negative mutant of JNK1 expression plasmid pcDNA3-FLAG-JNK1 (APF). Transfectants were exposed to glucose-free medium for various intervals (5–120 min) as indicated at the bottom of each lane. Western blot analysis was done as described in the legend to Fig. 6 with anti-ACTIVE JNK antibody or anti-actin antibody. C, untreated control cells.
induced JNK activation and subsequent HSP gene expression. Overexpression of the dominant-negative mutant of JNK1 also suppressed glucose deprivation-induced HSF activation as well as HSP70 gene expression (Figs. 10 and 11). These observations strongly suggest that JNK1 is involved in the downstream events of HSP70 gene expression which may be mediated through the activation of HSF. It is believed that the activation of HSF involves multiple steps (58, 59). In the first step, HSF assembles into a trimer after heat shock (60–62). Several researchers show that HSFs are multi-zipper proteins (61) and heat shock may alter interactions between the carboxyl-terminal zipper and the amino-terminal zipper. This alteration of intermolecular coiled-coil interactions results in the formation of HSF trimers (61, 62) which then accumulates within the nucleus (63–65) and binds to the HSE (36). The oligomeric state of the HSF that binds to an HSE is probably hexameric (66). The binding of HSF to HSE is necessary for transcriptional activation of eukaryotic hsp genes, however, HSF-DNA binding activity does not always correlate with transcriptional activity (67, 68). Moreover, in yeast cells HSF is already bound to the HSE prior to heat shock (5). These observations suggest that the simple binding of HSF to HSE is not sufficient for transcriptional activation. A modification of HSF, e.g. phosphorylation of HSF, is required to stimulate transcription (11, 69, 70). Okadaic acid, a potent and specific inhibitor of serine/threonine phosphatase 2A and 1, potentiates the heat-induced transcriptional activation of heat shock genes (71). Our data indicate that metabolic oxidative stress-activated JNK1 may be involved in HSF activation, perhaps phosphorylation of HSF. Interestingly, recent studies demonstrate that overexpression of hsp70 inhibits heat-induced SAPK signal transduction pathway activation as well as apoptosis (72, 73). These studies indicate that HSP70 is able to block apoptosis by inhibiting signaling events upstream of SAPK activation. Taken together, we suggest that HSP70 expression is itself a consequence of the activity of the SAPK pathway and overexpression of HSP70 inhibits the SAPK pathway through negative feedback regulation. Our proposed model will provide important insight to understand how heat shock or oxidative stress induces HSF activation and subsequently leads to HSP70 gene expression. This model will also provide a framework to study the critical steps in the regulation of hsp gene expression.

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