

10-Formyltetrahydrofolate Dehydrogenase–Induced c-Jun-NH₂-Kinase Pathways Diverge at the c-Jun-NH₂-Kinase Substrate Level in Cells with Different p53 Status

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Abstract

10-Formyltetrahydrofolate dehydrogenase (FDH) suppresses cancer cell proliferation through p53-dependent apoptosis but also induces strong cytotoxicity in p53-deficient prostate cells. In the present study, we have shown that FDH induces apoptosis in PC-3 prostate cells through simultaneous activation of the c-Jun-NH₂-kinase (JNK) and extracellular signal-regulated kinase (ERK) pathways with JNK phosphorylating c-Jun and ERK1/2 phosphorylating Elk-1. The JNK1/2 inhibitor SP600125 or ERK1/2 inhibitor PD98059 prevented phosphorylation of c-Jun and Elk-1, correspondingly and partially protected PC-3 cells from FDH-induced cytotoxicity. Combination of the two inhibitors produced an additive effect. The contribution from the JNK cascade to FDH-induced apoptosis was significantly stronger than from the ERK pathway. siRNA knockdown of JNK1/2 or “turning off” the downstream target c-Jun by either siRNA or expression of the dominant-negative c-Jun mutant, TAM67, rescued PC-3 cells from FDH-induced apoptosis. The pull-down assays on immobilized c-Jun showed that c-Jun is directly phosphorylated by JNK2 in FDH-expressing cells. Interestingly, the FDH-induced apoptosis in p53-proficient A549 cells also proceeds through activation of JNK1/2, but the down-stream target for JNK2 is p53 instead of c-Jun. Furthermore, in A549 cells, FDH activates caspase 9, whereas in PC-3 cells, it activates caspase 8. Our studies indicate that the JNK pathways are common downstream mechanisms of FDH-induced cytotoxicity in different cell types, whereas the end point target in the cascade is cell type specific. JNK activation in response to FDH was inhibited by high supplementation of reduced folate leucovorin, further indicating a functional connection between folate metabolism and mitogen-activated protein kinase pathways. (Mol Cancer Res 2009;7(1):99–107)

Introduction

c-Jun NH₂ terminal kinase/stress-activated protein kinases (JNK; ref. 1) are members of the family of mitogen-activated protein kinases (MAPK), which also includes extracellular signal-regulated kinases (ERK) and p38 kinase (2). MAPKs are activated by variety of extracellular and intracellular stimuli and control a diverse array of cellular functions such as gene expression, inflammatory response, differentiation, cell cycle, cell proliferation, and apoptosis (3, 4). Earlier studies correlated stress-related activation of JNK and p38 with induction of apoptosis, whereas activation of ERK pathways in response to cytokines and growth factors was primarily implicated in mitogenic and antiapoptotic effects (3). Numerous later studies resulted in the revision of this simplified view and provided more integrated picture in which the specific role of MAPKs in cellular processes depends on the stress signal and the cell type (5-7). In accordance with this concept, both prosurvival and proapoptotic effects of JNKs have been reported (8). A role of JNKs in induction of apoptosis is well-established: activation of the JNK pathway in response to various stress stimuli has been shown to lead to apoptosis in cell culture and animal models, whereas interruption of the JNK pathway suppresses apoptosis in many cases (3, 9-11). However, effects of JNK on apoptotic signaling are cell type dependent rather than universal (reviewed in ref. 12).

JNKs phosphorylate and activate transcription factor c-Jun (13), which regulates broad spectrum of events including apoptotic cell death and oncogenic transformation. In recent years, many other proteins were identified as JNKs targets (reviewed in refs. 14, 15). The list of JNKs substrates includes numerous transcription factors, hormone receptors, histones, members of Bcl-2 family of apoptosis-related proteins, the E3 ligase Itch, and components of cell motility machinery (reviewed in ref. 14). Such a diversity of the JNK substrates together with existence of three JNK genes and multiple splice variants (13, 16, 17), could explain complex and sometime opposite effects of the JNK pathways as well as the cell-type specificity. The net effect of JNK activation also depends on the signaling pathways that are simultaneously activated (12). In this context, it should be noted that different MAPKs can act upon the same protein targets: for example, two common JNK substrates, transcription factors activating transcription factor (ATF)-2 (18) and Elk-1 (19), are also canonical substrates for p38 and ERK MAPKs. At the same time, numerous studies indicate that phosphorylation of c-Jun *in vivo* is selectively catalyzed by JNKs (20).

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JNK pathways are activated in response to a variety of stress stimuli, including UV irradiation, DNA damage, heat shock and oxidants, as well as inflammatory cytokines (21, 22). We have recently shown that elevation of a folate enzyme, 10-formyltetrahydrofolate dehydrogenase (FDH), in A549 cells activates an apoptotic pathway, in which the p53 tumor suppressor is phosphorylated by JNK2 at Ser6 (23). Most cancer cells are FDH-deficient and elevation of the enzyme in these cells produces strong cytotoxic effects including suppression of proliferation and apoptosis (24-26). Potential mechanisms of FDH-induced cellular stress are as follows: inhibition of *de novo* purine biosynthesis (26), altered methylation processes (27), and overall limitation of carbon units in the folate pool (28). An additional mechanism could be an increase of oxidative stress because the FDH substrate, 10-formyltetrahydrofolate, has been proposed to serve as an important cellular antioxidant (29). In A549 cell line, as well as in HCT-116 cells, both of which are p53 proficient, the FDH-induced suppressor effects are strictly p53-dependent (25, 26). At the same time, FDH is cytotoxic in p53-deficient cell lines as well (24). The pathways through which FDH operates in these cells

were not clear. In the present study, we examined the mechanisms of FDH-induced apoptosis in p53-null prostate cell line PC-3 and showed that FDH still activates the JNKs pathway in these cells but it is diverted to the phosphorylation of c-Jun instead of p53.

Results

FDH Induces Apoptosis in PC-3 Prostate Cells

We have previously observed that FDH has strong cytotoxic effects on numerous cancer cell lines, including androgen-independent p53-null PC-3 prostate cells (24). To determine whether FDH induces apoptosis in these cells, we transiently transfected them with pcDNA3.1/FDH construct. Western blot analysis indicated appearance of FDH 24 hours posttransfection and its levels remained constant up to 5 days posttransfection (Fig. 1A). Simultaneously, proliferation of FDH expressing cells was strongly inhibited (Fig. 1A). Expression of catalytically inactive C707A FDH mutant (24) did not inhibit proliferation (Fig. 1A), indicating specificity of the antiproliferative effects of catalytically active FDH. The induction of

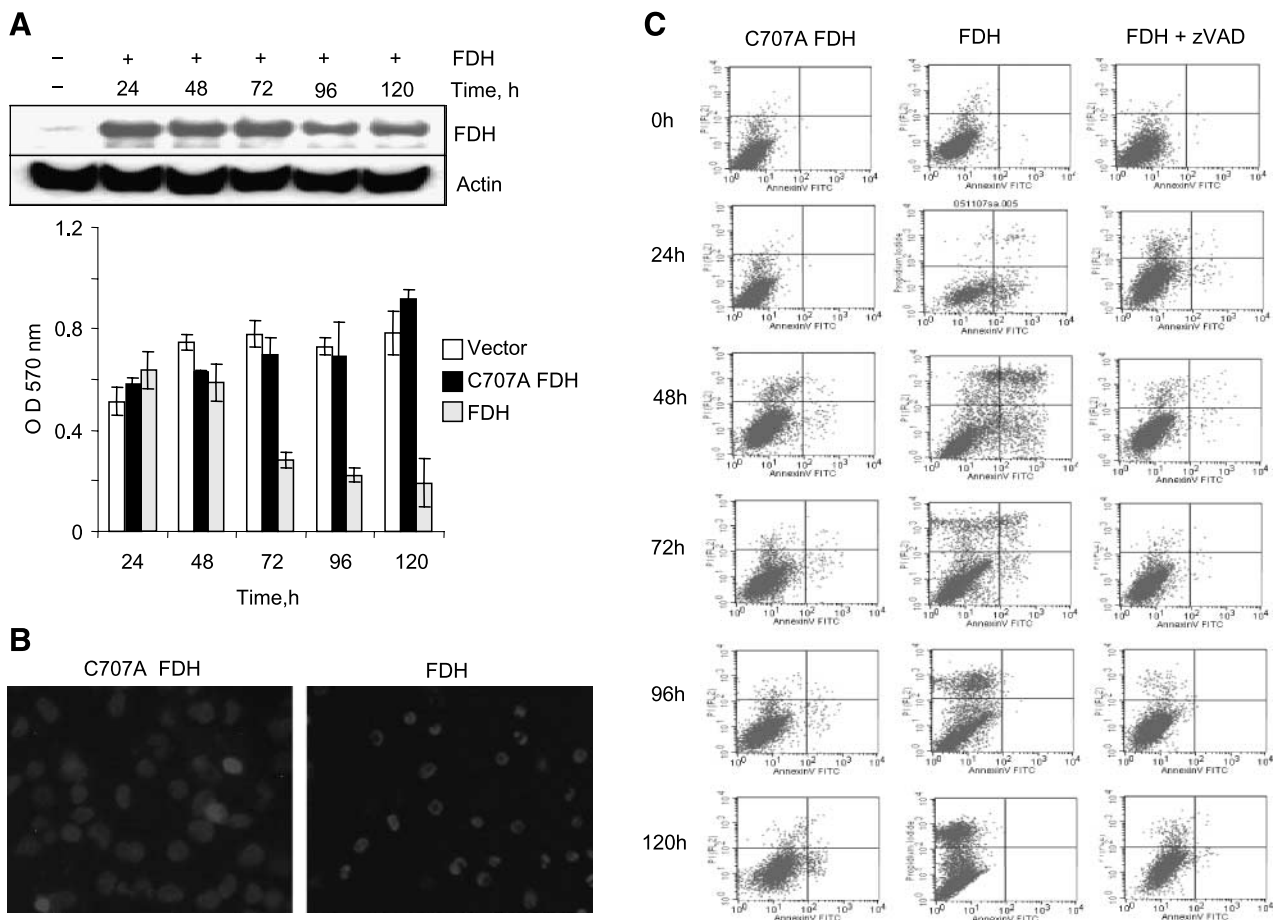


FIGURE 1. FDH antiproliferative effects in PC-3 cells. **A.** Viable cells assessed by MTT assay at indicated time points after FDH expression (gray bars); C707A mutant expression (black bars); or after transfection with empty vector (control; open bars). Inset, FDH levels evaluated by immunoblot. **B.** Fluorescence microscopy of Hoechst stained cells expressing FDH or its C707 mutant. Cells were incubated with Hoechst 33258 (2.5 μ g/mL) for 10 min and examined using Olympus IX70 fluorescent microscope. **C.** Apoptosis detected by fluorescence activated cell sorting flow cytometry in PC-3 cells. Left, (control), cells transfected for inactive C707A FDH expression; middle, cells transfected for FDH expression; right, cells transfected for FDH expression in the presence z-VAD-fmk. Time posttransfection is indicated.

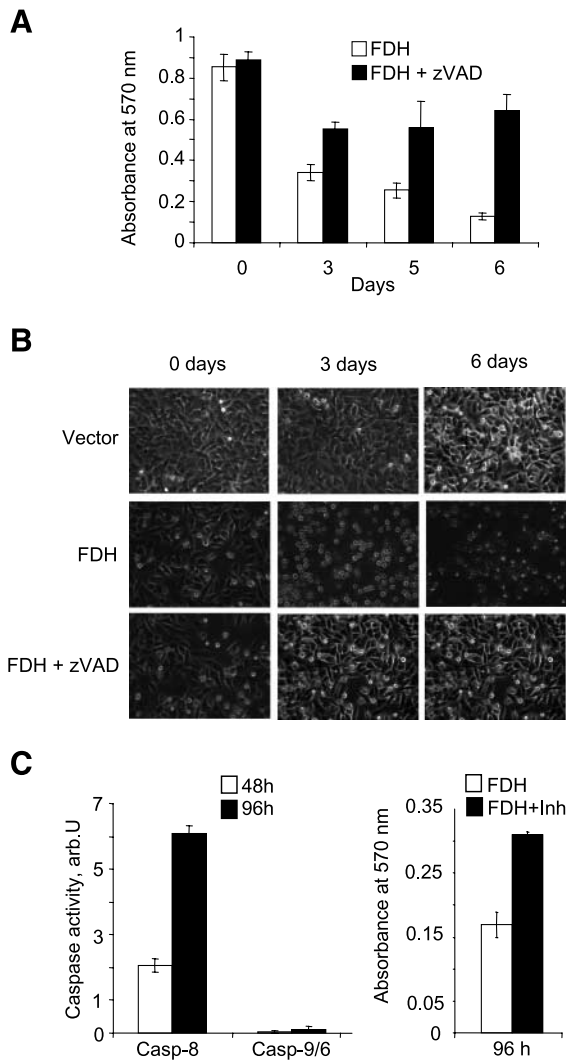


FIGURE 2. Caspase inhibitors protect PC-3 cells from FDH suppressor effects. **A.** Proliferation of FDH expressing cells in the presence (black bars) and in the absence (open bars) of z-VAD-fmk assessed by MTT assays. **B.** Phase-contrast microscopy of “empty” vector-transfected cells (top), FDH-transfected cells (middle), and FDH-transfected cells in the presence of z-VAD-fmk (bottom). **C.** Activities of caspase 8 and 9/6 in FDH-expressing cells (left) and proliferation of the cells in the presence of the caspase 8 inhibitor z-IETD-fmk assessed by MTT assays (right). FDH posttransfection time is indicated.

apoptosis in FDH-expressing cells was evident from the cell morphology: Hoechst-stained cells exhibited condensed and fragmented nuclei (Fig. 1B). Annexin V assay has further confirmed apoptosis, which was observed as early as 24 hours posttransfection (Fig. 1C).

The pan-caspase inhibitor z-VAD-fmk effectively protected PC-3 cells against FDH-induced cytotoxicity at 50 $\mu\text{mol/L}$ concentration (Fig. 2A). z-VAD-treated FDH-expressing cells displayed morphologic characteristics of nonapoptotic cells in contrast to FDH-expressing nontreated cells, in which apoptotic morphology was clearly seen (Fig. 2B). In agreement with these data, Annexin V assays showed a strong suppression of FDH-induced apoptosis in presence of z-VAD-fmk (Fig. 1C).

Caspase assays have shown strong increase of caspase 8 activity in FDH-expressing PC-3 cells, whereas caspase 9 was not activated in these cells (Fig. 2C). In agreement with these data, treatment of cells with the caspase 8-specific inhibitor z-IETD-fmk (30) protected them from antiproliferative effects of FDH at 30 $\mu\text{mol/L}$ concentration (Fig. 2C).

FDH Activates JNK and ERK in PC-3 Cells

To investigate whether FDH-induced apoptosis is mediated via MAPK signaling pathways, we assessed phosphorylation of JNK1/2, ERK1/2, and p38 in FDH-transfected cells. FDH expression resulted in strong phosphorylation of JNK1/2 and ERK1/2 but not p38 (Fig. 3A). Phosphorylation of the kinases became prominent at 48 hours posttransfection and stayed at maximum level till 96 hours posttransfection. At the same time, basal levels of kinases themselves did not change (Fig. 3A). We have also examined levels of phosphorylated c-Jun, Elk-1, and ATF-2, canonical targets of MAPKs. Two of these proteins, c-Jun and Elk-1, showed higher levels of phosphorylated form at 48 to 72 hours FDH posttransfection, whereas phosphorylation of ATF-2 was not changed (Fig. 3B).

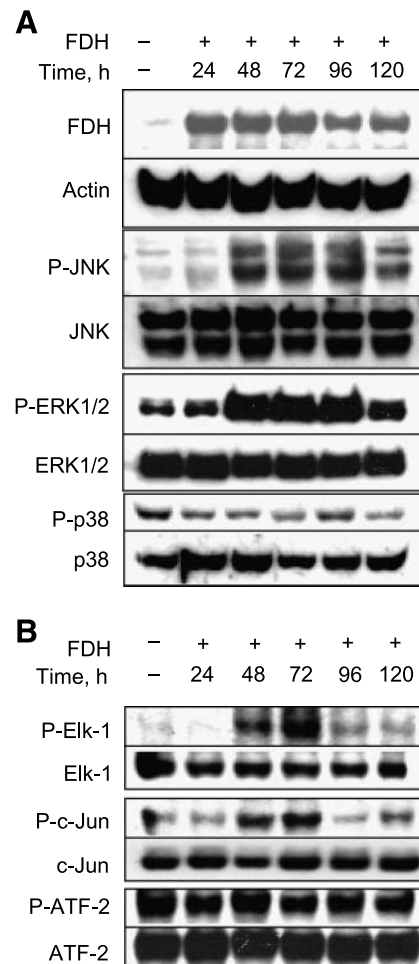


FIGURE 3. FDH induces phosphorylation of JNK1/2 (A), and c-Jun and Elk-1 (B) in PC-3 cells. FDH posttransfection time is indicated.

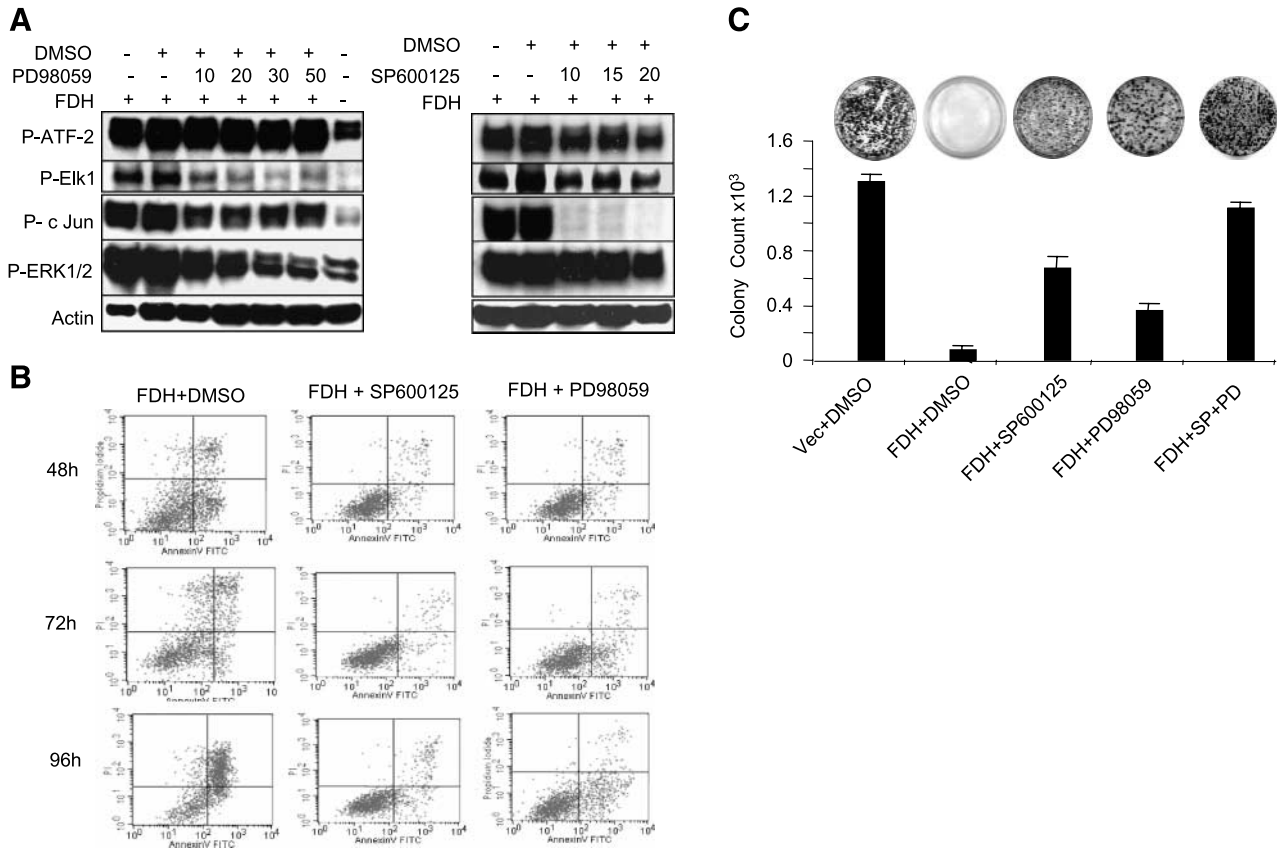


FIGURE 4. MAPK inhibitors protect cells against FDH-induced apoptosis. **A.** Effects of the ERK1/2 inhibitor PD98059 and the JNK inhibitor SP600125 on phosphorylation of MAPKs and their substrates in FDH-expressing cells. Concentrations of inhibitors ($\mu\text{mol/L}$) are indicated. **B.** Apoptosis detected by fluorescence activated cell sorting flow cytometry in FDH expressing PC-3 cells: in the absence of MAPK inhibitors (*left*); in the presence of $10 \mu\text{mol/L}$ of the JNK inhibitor SP600125 (*middle*); in the presence of $30 \mu\text{mol/L}$ the ERK1/2 inhibitor (*right*). Time of FDH posttransfection is indicated. **C.** Colony formation by PC-3 cells expressing FDH and treated with SP600125, PD98059, or both simultaneously.

MAPK Inhibitors Rescue PC-3 Cells from FDH-induced Apoptosis

To examine the specific role of JNK1/2 and ERK1/2 in PC-3 cells in response to FDH, we inhibited these kinases with the specific inhibitors SP600125 and PD98059, correspondingly. The JNK inhibitor completely blocked phosphorylation of c-Jun at concentration of $10 \mu\text{mol/L}$, whereas the ERK inhibitor significantly decreased levels of phosphorylated Elk-1 at concentrations 10 to $50 \mu\text{mol/L}$ (Fig. 4A). The level of phosphorylated ATF-2 was not affected by either of these inhibitors (Fig. 4A). In agreement with the decreased levels of phosphorylated c-Jun and Elk-1, we observed the decrease in number of apoptotic cells after FDH expression (Fig. 4B). However, protection from apoptosis was much more profound with the JNK inhibitor than with the ERK inhibitor as was seen in the clonogenic assays (Fig. 4C): survival rate compared with the control nontreated cells was 50% and 28%, correspondingly. Combination of the two inhibitors produced the additive effect with almost quantitative outcome (Fig. 4C). We have also observed the decrease in levels of phosphorylated ERK1/2 in the cells treated with PD98059 (Fig. 4A). Such effects, however, have been reported and they are likely the result of the inhibition of the ERK upstream kinase MAPKK1 (31, 32).

siRNA Knockdown of JNKs Protects PC-3 Cell from FDH Suppressor Effects

The experiments with the JNK inhibitor indicated that the JNK pathways are important for the response to FDH-related stress in PC-3 cells. This conclusion was further confirmed in experiments with siRNA knockdown of JNKs. Both JNK1 and JNK2 were simultaneously targeted in these experiments. As can be seen in Fig. 5A and B, the knockdown was efficient with the strong decrease in the levels of mRNA and the protein for both JNK1 and JNK2. Cell viability assessed by trypan blue exclusion as well as clonogenic assay showed that JNK knockdown protects PC-3 cells against FDH suppressor effects (Fig. 5C and D).

c-Jun Is the Downstream Mediator of FDH Stress in PC-3 Cells

Phosphorylation of the canonical JNK target, c-Jun, in PC-3 cells in response to FDH (Fig. 3) implies its role in FDH effects. To study whether disabling c-Jun will block antiproliferative effects of FDH in these cells, we have expressed the dominant-negative truncated mutant of c-Jun, TAM67, which inhibits the c-Jun-mediated signaling pathways (33). We have observed

that expression of TAM67 significantly decreased FDH antiproliferative effects in this cell line (Fig. 6A and B), supporting the crucial function of c-Jun in response to FDH-induced stress. Additional support of the role of c-Jun in this system came from experiments with siRNA knockdown of c-Jun, in which strong silencing of the protein was achieved using Steals RNA duplex (Fig. 6C). We have observed that, similarly to TAM67 mutant, the c-Jun silencing strongly protected PC-3 cells against FDH cytotoxicity (Fig. 6D and E).

c-Jun Is Phosphorylated by JNK2 in Response to FDH in PC-3 Cells

We have previously shown that, in A549 cells, FDH induces a cascade in which JNK2 is the end point kinase phosphorylating p53 at Ser6 (23). In the present study, we have found that JNK2 is also the ultimate kinase of the cascade in PC-3 cells but it uses a different substrate, c-Jun. We have used c-Jun immobilized on agarose beads to pull down the kinase that is responsible for c-Jun phosphorylation. These experiments have shown that phosphorylated JNK2 but not JNK1 was pulled down from PC-3 cells after expression of FDH (Fig. 7A). In the absence of FDH, there was no JNK2 detected in the pulled down sample (Fig. 7A). In agreement with these results, *in vitro* kinase assay has shown that c-Jun was phosphorylated *in vitro* by JNK2 pulled down from FDH-expressing cells (Fig. 7B).

Leucovorin Supplementation Inhibits FDH-Mediated JNK Activation

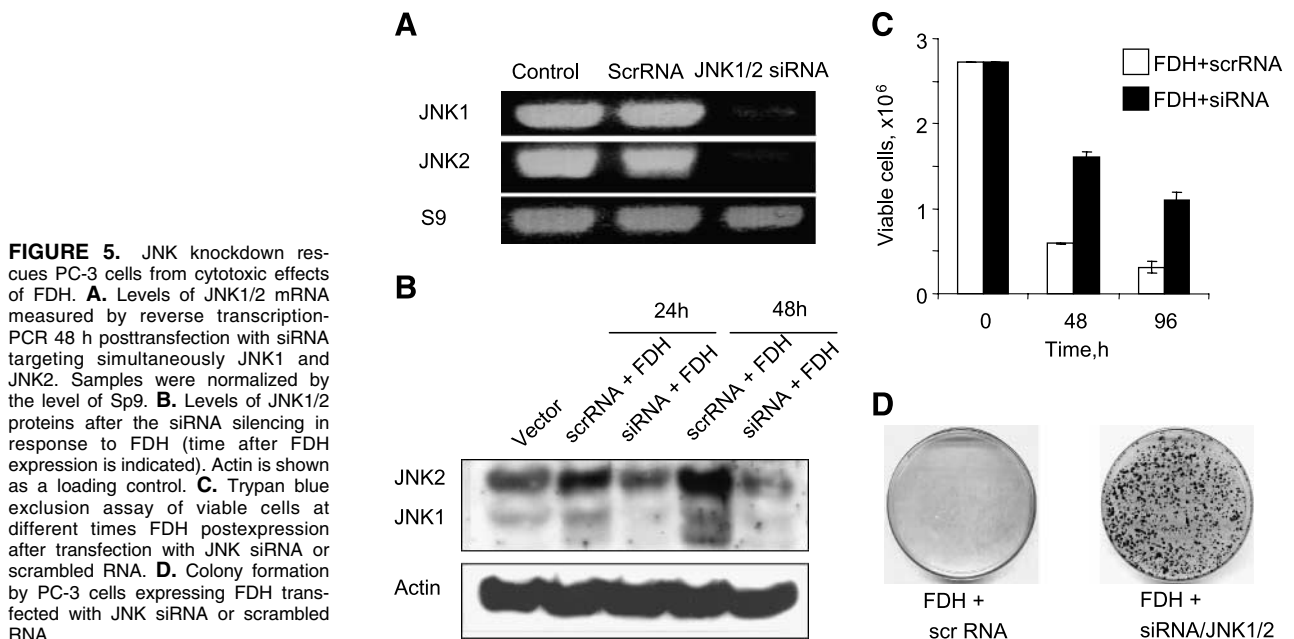
FDH suppressor effects can be reversed by supplementation of high concentrations of reduced folate leucovorin (5-formyltetrahydrofolate; refs. 24, 34). To investigate whether the protective effects of leucovorin proceed through inhibition of the JNK pathway, we evaluated JNK phosphorylation in FDH-expressing PC-3 and A549 cells growing on medium

containing 10 $\mu\text{mol/L}$ of this reduced folate. We observed that leucovorin strongly inhibited FDH-induced phosphorylation of JNK in both cell lines (Fig. 8). We also observed the lack of phosphorylation of c-Jun in PC-3 cells and Ser6 of p53 in A549 cells in these experiments (Fig. 8). Another MAPK pathway activated by FDH in PC-3 cells, ERK, was inhibited as well: leucovorin resulted in complete abolishing phosphorylation of both ERK and its substrate Elk-1, whereas they are strongly phosphorylated in the absence of this supplementation (Fig. 8A).

Discussion

We have previously shown that FDH induces p53-dependent apoptosis in p53-proficient cell lines (25, 26). In A549 cells, this apoptosis proceeds through phosphorylation of p53 at Ser6 by JNKs, whereas the canonical JNK target, c-Jun, was not phosphorylated in these cells in response to FDH (23). The fact that JNK apoptotic pathway can proceed in bypass of c-Jun is not unusual. For example, JNK-dependent apoptosis in the absence of c-Jun phosphorylation was observed in A549 cells in response to ceramides (35). In addition, JNK-independent phosphorylation of c-Jun can be a prosurvival factor in these cells (36).

Surprisingly, whereas disabling the p53 pathways in p53-proficient cells protects them from FDH-induced apoptosis and associated cytotoxicity (26), at the same time, FDH induces antiproliferative effects in p53-deficient prostate cancer cell lines (24). This puzzling observation prompted us to investigate mechanisms of cytotoxicity in response to FDH in these cells. Because tumor suppressor p53 is mutated in >50% of human cancers (37), clarification of this pathway might have important implications. Potentially, FDH as a metabolic regulator could induce nonapoptotic antiproliferative effects through depletion of important cellular metabolites. Indeed, strong decrease of



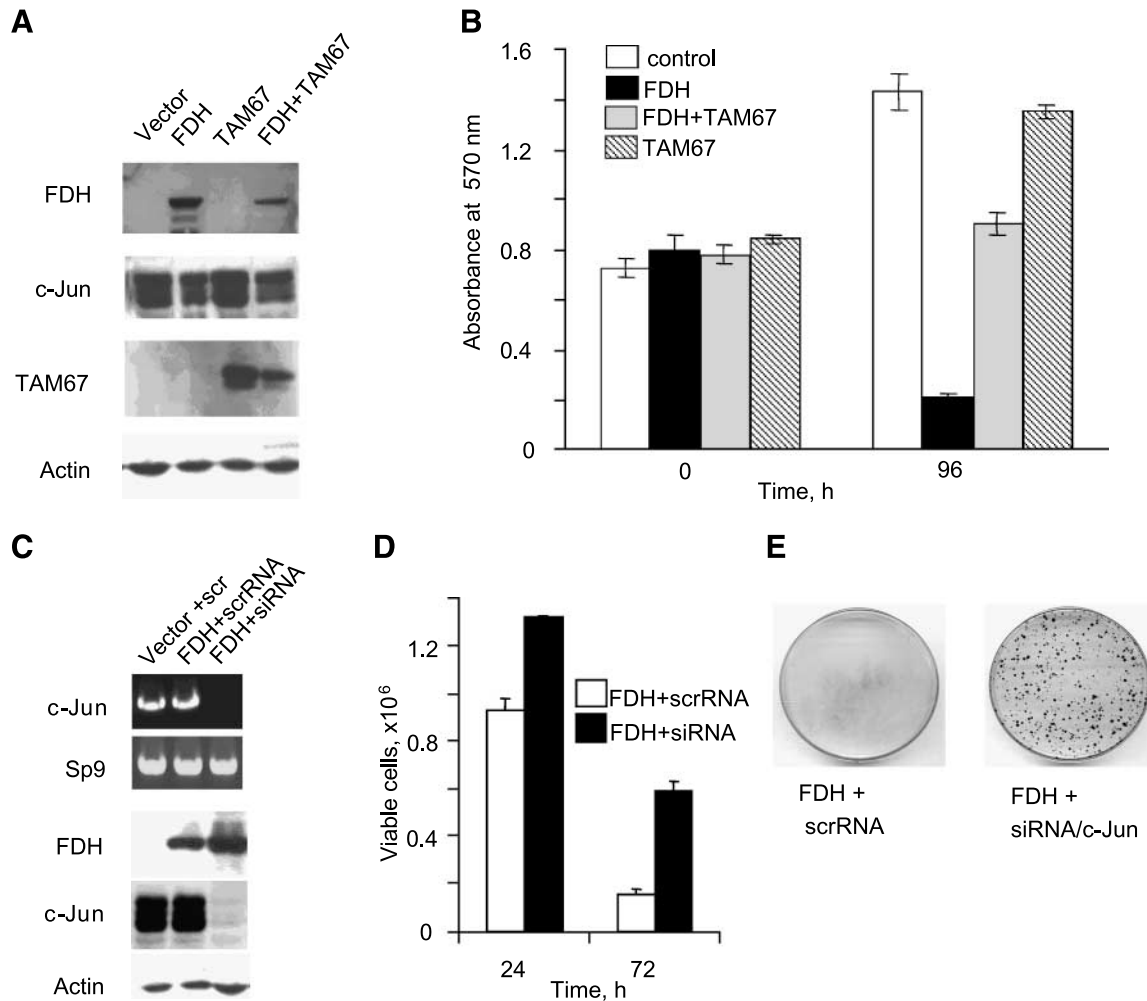


FIGURE 6. c-Jun is the downstream target of JNK1/2 in the FDH-induced apoptotic pathway in PC-3 cells. **A.** Levels of FDH, c-Jun, and TAM67 after transient expression in PC-3 cells evaluated by immunoblotting. Actin is shown as a loading control. **B.** MTT assay of PC-3 cells expressing FDH, TAM67, or both simultaneously. **C.** siRNA silencing of c-Jun in FDH-expressing PC-3 cells (*top*); levels of c-Jun mRNA measured by reverse transcription-PCR, samples were normalized for Sp9; *bottom*, levels of FDH and c-Jun proteins assessed by immunoblot, actin is shown as a loading control). **D.** Trypan blue exclusion assay of viable cells at different times FDH postexpression after transfection with c-Jun siRNA or scrambled RNA. **E.** Colony formation by PC-3 cells expressing FDH transfected with c-Jun siRNA or scrambled RNA.

FDH substrate, 10-formyltetrahydrofolate and associated depletion of ATP/GTP were observed in FDH-expressing cells (26, 34). We found, however, that similar to the p53-proficient cells, FDH induces apoptosis in p53-deficient PC-3 cells. Moreover, the apoptosis in these cells also proceeds through activation of the JNK pathways as in A549 cells. In contrast to A549 cells, JNK phosphorylates c-Jun in response to FDH in PC-3 cells. Another difference with A549 cells was activation of ERK1/2 in PC-3 cells. Interestingly, in contrast to A549 cells, in which FDH-induced apoptosis is associated with activation of caspase 9 (25), in PC-3 cells, this caspase was not active. Instead, activity of caspase 8 was strongly up-regulated in these cells in response to FDH. This is in agreement with several reports, which showed connection between c-Jun/JNK pathway and caspase 8, but not caspase 9, activation in several cell lines (38-40). Of note, such a phenomenon was observed in PC-3 cells, in which JNK activation seems to be upstream of

caspase 8 (38). In support of this conclusion, it has been recently shown that caspase 8 promoter has a c-Jun/AP1 regulatory site, which controls transcription in response to mitomycin C in Huh7 hepatoma cells (41).

In general, the JNK pathways are associated with phosphorylation of c-Jun that protects it from ubiquitine/proteasome degradation (42) and activates its function as a transcription factor (17, 43). Activation of JNKs and associated phosphorylation of c-Jun is a common event in PC-3 cells although final outcome can be either proapoptotic (44, 45) or prosurvival (46, 47). JNKs can also act upon such downstream targets as ATF-2 (18) and Elk-1 (19), which are substrates for other MAPKs (48). In our studies, JNK was responsible for c-Jun phosphorylation only, whereas Elk-1 was simultaneously phosphorylated by ERK1/2. Although ERK1/2 activation is commonly associated with cell proliferation and is viewed as antiapoptotic factor, prolonged activation of ERK1/2 is often responsible for

cell death (49). In PC-3 cells, for example, the ERK dependent apoptosis was shown in response to phenylethyl isothiocyanate treatment (50). Apparently, the crosstalk between JNK and ERK pathway is a common phenomenon in PC-3 cells: several studies have shown that apoptosis in these cells proceeds through simultaneous activation of JNK and ERK but not p38 (51-54). Similarly, JNK and ERK pathways were simultaneously activated in response to FDH in these cells. The lack of a synergistic effect between these two pathways in our experiments implies that they operate independently in response to FDH stress. Stronger contribution of JNKs to FDH-induced apoptosis and the previously established role of this pathway in FDH signaling in A549 cells (23) indicate that JNKs are the main downstream effectors of FDH and a common mechanism of its cytotoxicity in different types of cancer.

Further evidence for the conclusion regarding universal role of JNK in mediation of FDH cytotoxicity came from experiments with supplementation of FDH-expressing cells with the reduced folate leucovorin (5-formyltetrahydrofolate). We have earlier shown that high concentration of leucovorin in culture medium protected cells against FDH and produced clones, which are FDH resistant (24, 34). Mechanism of the immediate protection by leucovorin supplementation is related to a strong increase of intracellular pool of reduced folates with simultaneous inhibition of FDH by the excess of tetrahydrofolate, the product of FDH-catalyzed reaction (34). Of note, the protective effect of leucovorin was observed in both PC-3 and A549 cell lines (24). Therefore, it would be expected that, if activation of JNK and phosphorylation of its substrates is the major downstream event in apoptotic response to FDH, this cascade should be disabled in protected cells despite of their origin. This was found to be the case: leucovorin prevented phosphorylation of

JNK in both cell lines. In accordance with this finding, it also prevented phosphorylation of JNK targets, c-Jun and p53, in PC-3 and A549 cells, correspondingly. Interestingly, inhibition of the ERK component of FDH response was further observed in PC-3 cells, supporting our conclusion that this pathway plays a proapoptotic role upon FDH expression in these cells.

In conclusion, the present study showed that activation of JNK pathways is a common downstream event in FDH-induced cytotoxicity and answered the question of how FDH can induce apoptosis in p53-deficient cells. It also indicates that, depending on the cell type, the FDH-induced JNK signaling can diverge at the level of JNK substrate. In PC-3 cells, for example, this pathway proceeds through phosphorylation of a canonical JNK substrate, c-Jun, whereas in A549 cells, c-Jun is not a target for FDH-induced JNKs. It is not clear at present what switches JNK activity to a different substrate in response to FDH. Obviously, availability of additional factors, such as scaffold proteins, or coactivation of additional pathways could contribute to this mechanism. For example, in PC-3 cells FDH induces ERK1/2, which is not the case in A549 cells. Of note, simultaneous activation of JNK/c-Jun and ERK/Elk-1 pathways is a common apoptotic response in PC-3 cells. Our study also indicates a functional connection between folate metabolism and MAPK pathways, although precise mechanisms of how folate regulates these pathways await further investigations.

Materials and Methods

Reagents

MAPK inhibitors were obtained from Calbiochem (PD98059) and Sigma (SP600125). Caspase inhibitors were from Calbiochem. Stock solutions of the inhibitors were prepared in DMSO. Inhibitors were added to the cell culture 24 h FDH posttransfection. Medium containing inhibitors was replaced every 48 h.

Cell Culture

The prostate adenocarcinoma cell line PC-3 was obtained from American Type Culture Collection. Non-small cell lung carcinoma cell line A549 capable of Tet-On inducible expression of FDH was generated in our previous studies (25). Cell culture medium and reagents were purchased from Invitrogen. Cells were maintained in RPMI 1640 supplemented with 10% fetal bovine serum, 2 mmol/L glutamine, and 1 mmol/L sodium pyruvate (complete medium) at 37°C under humidified air containing 5% CO₂. Cell viability was assessed by trypan blue exclusion or by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell proliferation assay using cell Titer 96 kit (Promega).

Transfection

PC-3 cells ($\sim 1.4 \times 10^6$) were transfected with pcDNA3.1/FDH vector (2 μ g) or cotransfected with pcDNA3.1/FDH vector (1 μ g) and pcDNA3.1/TAM67 (1 μ g; a kind gift from Dr. Michael Birrer, National Cancer Institute, NIH, Rockville, MD) using Amaxa electroporation kit "V" according to the manufacturer's directions. To allow the selection of transfected cells, 500 μ g/mL of G418 (Sigma) was added to the culture 24 h posttransfection. FDH and TAM67 expression was detected by immunoblot assays.

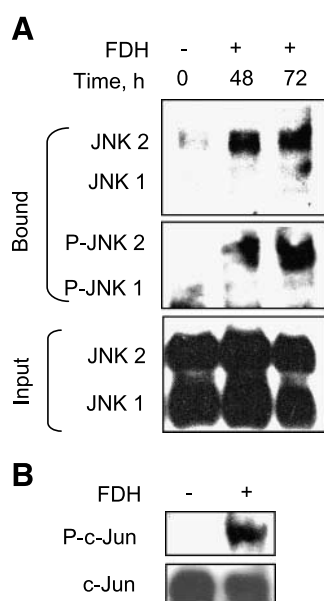


FIGURE 7. c-Jun is phosphorylated by JNK2 in response to FDH in PC-3 cells. **A.** Levels of JNK1 and JNK2 and their phosphorylated forms pulled down from FDH-expressing cells on immobilized c-Jun. **B.** *In vitro* kinase assay showing phosphorylation of c-Jun by the kinase pulled down from FDH-expressing cells.

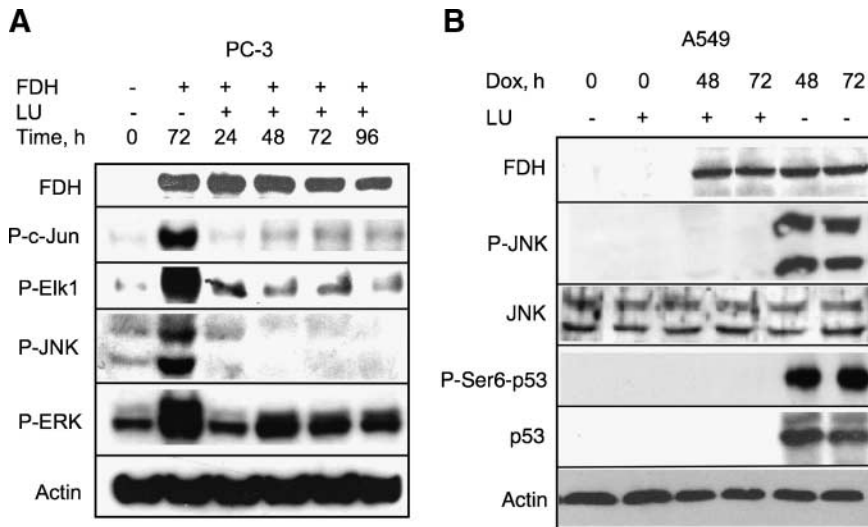


FIGURE 8. High leucovorin supplementation prevents activation of MAPK pathways in response to FDH. **A.** Levels of phosphorylated JNK, ERK, c-Jun, and Elk-1 in PC-3 cells in the presence and in the absence of leucovorin (LU) supplementation. **B.** Levels of phosphorylated JNK and Ser6 p53 in A549 cells in the presence and in the absence of leucovorin supplementation. Expression of FDH in Tet-On A549/FDH cells was induced by addition of 2.5 $\mu\text{g}/\text{mL}$ doxycycline.

Immunoblot Analysis

Western blot analysis was done using cell lysate prepared in buffer containing 50 mmol/L Tris-HCl buffer (pH 8.0), 150 mmol/L NaCl, 2 mmol/L EDTA, 1% Triton X-100, 0.1% SDS, 1 mmol/L DTT, 1 mmol/L phenylmethylsulfonyl fluoride, and protease inhibitor cocktail (Sigma). Cell lysates were subjected to SDS-PAGE followed by immunoblot with corresponding antibodies.

Antibodies

FDH was detected using in-house FDH-specific polyclonal antiserum (1:5,000); actin was detected using monoclonal antibodies (1:5,000; Calbiochem). Stress-activated protein kinase/JNK, Phos-SAPK/JNK (Thr183/Tyr185), Phospho-ERK1/2 (Thr202/Tyr204), Phospho-p38 (Thr180/Tyr182), ATF-2, Phospho-ATF-2 (Thr71), c-Jun, Phospho-c-Jun (Ser63), Elk-1, and Phospho-Elk-1(Ser383) were detected using antibodies from Cell Signaling Technology (1:1,000). Antibody for ERK1/2, and p38 were purchased from Chemicon (1:1,000).

In vitro Kinase Assay

In vitro assay of JNK was done using MAPK assay kit (Cell Signaling Technology) according to manufacturer's manual. Kinase activities were evaluated based on the phosphorylation of c-Jun (12) that was assessed by immunoblot with specific antibodies.

Apoptosis Assay

Apoptosis was detected by Annexin V labeling using Annexin V-FLUOS staining kit (Roche Molecular Biochemicals). Activities of caspase 8 and caspase 9/6 were assayed by ApoAlert caspase assay kit (Becton Dickinson).

siRNA

PC-3 cells (1.4×10^6) were cotransfected with 65 nmol/L of the siRNA duplex (targeting the sequence 5'-AAAGAAU-GUCCUACCUUCU-3' identical in JNK1 and JNK2; ref. 55) and 2 μg of pcDNA3.1/FDH vector using AMAXA electro-

poration kit "V". Silencing of c-Jun was carried out in a similar manner using 75 nmol/L of Stealth RNAi (Invitrogen). Two c-Jun target sequences, 5'-GCAAACCUCAGCAACUCAA-CCCAG-3' and 5'-GCAAAGAUGGAAACGACCUUCUA-UG-3', were used in these experiments with similar outcome. Scrambled Stealth RNAi (Invitrogen) was used as a negative control. Block-iT fluorescent oligo indicated a high efficiency of transfection. JNK1 and JNK2 or c-Jun mRNA and protein levels were evaluated by reverse transcription-PCR and immunoblot, correspondingly. Total RNA for reverse transcription-PCR was isolated using RNA Easy Protect Mini kit (Qiagen). Reverse transcriptase reaction was done with oligo (dT)₁₈ primer using BD SMART cDNA synthesis kit (Clontech). Conventional PCR was carried out using specific primers and Pfu-Turbo DNA polymerase (Stratagene) as described (23).

Clonogenic Assay

Colony formation by PC-3 cells after treatment with MAPK inhibitors or siRNA knockdown of JNK or c-Jun were assessed 2 to 3 wk FDH posttransfection. Colonies were visualized by staining with 0.2% crystal violet. The number of colonies was analyzed using Quantity One software (Bio-Rad).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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