Cross-talk between Endoplasmic Reticulum (ER) Stress and the MEK/ERK Pathway Potentiates Apoptosis in Human Triple Negative Breast Carcinoma Cells

ROLE OF A DIHYDROPYRIMIDONE, NIFETEPIMINE

Received for publication, July 3, 2014, and in revised form, December 15, 2014 Published, JBC Papers in Press, December 19, 2014, DOI 10.1074/jbc.M114.594028

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Background: Triple negative breast cancers (TNBC) are resistant to conventional therapies and molecular targets of TNBC are not explored in detail.

Results: Coupling of ER stress and the MEK/ERK pathway by nifetepimine effectively kills TNBC cells.

Conclusion: Nifetepimine plays a significant role in inducing apoptosis in TNBC cells.

Significance: Our findings strongly suggest that nifetepimine may act as a potent anti-cancer agent for treatment of TNBC.

Triple negative breast cancers (TNBC) are among the most aggressive and therapy-resistant breast tumors and currently possess almost no molecular targets for therapeutic options in this horizon. In the present study we discerned the molecular mechanisms of potential interaction between the endoplasmic reticulum (ER) stress response and the MEK/ERK pathway in inducing apoptosis in TNBC cells. Here we observed that induction of ER stress alone was not sufficient to trigger significant apoptosis but simultaneous inhibition of the MEK/ERK pathway enhanced ER stress-induced apoptosis via a caspase-dependent mechanism. Our study also demonstrated nifetepimine, a dihydropyrimidine derivative as a potent anti-cancer agent in TNBC cells. Nifetepimine down-regulated the MEK/ERK pathway in MDAMB-231 and MDAMB-468 cells and resulted in blockage of ER stress-mediated GRP78 up-regulation. Detailed mechanistic studies also revealed that nifetepimine by down-regulating pERK expression also declined the promoter binding activity of TFII-I to the GRP78 promoter and in turn regulated GRP78 transcription. Studies further extended to in vivo Swiss albino and SCID mice models also revalidated the anti-carcinogenic property of nifetepimine. Thus our findings cumulatively suggest that nifetepimine couples two distinct signaling pathways to induce the apoptotic death cascade in TNBC cells and raises the possibility for the use of nifetepimine as a potent anti-cancer agent with strong immune-restoring properties for therapeutic intervention for this group of cancer bearers.

Triple negative breast cancer (TNBC) is a subtype of breast cancer that lacks estrogen receptor and progesterone receptor expression, and does not overexpress epidermal growth factor receptor 2 (Her2/Neu) (1–3). Approximately 15% of globally diagnosed breast cancers are designated as estrogen (ER)-, progesterone-, and Her2/neu-negative (4–6). Studies have shown that tumors of this aggressive subtype are of higher histological grade, and are more likely to recur earlier at distant sites, resulting in poor overall prognoses (1, 2, 7, 8). To improve outcomes of TNBC, one must unravel the biological pathways operating and the modes of progression of TNBC and utilize this knowledge in improving the present day therapy regimes.

Currently, investigating endoplasmic reticulum stress as a potential target for cancer therapy has gained importance for exploring new horizons in drug development for these subgroups of breast cancer patients. The efficient functioning of the ER is essential for proper cellular activities and survival. Several cellular stress conditions, such as nutrient deprivation, hypoxia, and disturbances of calcium flux, lead to accumulation and aggregation of unfolded and/or misfolded proteins in the ER lumen and cause so-called ER stress (9–11). The ER responds to stress conditions by activation of a range of stress response signaling pathways, which couples the ER protein folding load with the ER protein folding capacity and is termed the unfolded protein response (UPR).

The microenvironment of cancerous cells and solid tumors is characterized by an inherent physiological ER stress response that provides an overall protective role in tumor development (e.g. elevated expression of GRP78 has been reported in several cancers, such as breast cancer and prostate cancer) (12–15). Moreover, GRP78 expression has been shown in some cases to be associated with tumor development and growth and corre-

The abbreviations used are: TNBC, triple negative breast cancer; pERK, phospho-extracellular signal regulated kinase; GRP78, glucose-regulated protein 78; EAC, Ehrlich's ascites carcinoma; PI, propidium iodide; ER, endoplasmic reticulum; UPR, unfolded protein response.

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lated with resistance to certain forms of chemotherapy. It seems that some cancer cells may have adapted to ER stress by activation of the UPR without resulting in apoptosis (13, 14, 16). As a “master” regulator of the UPR, GRP78 is believed to play an essential role in counteracting the apoptosis inducing potential of ER stress by multiple mechanisms such as binding to the unfolded proteins to alleviate ER stress conditions, and binding to calcium to prevent its release from the ER (12). Reports suggest that human caspase-4 plays an important role in ER stress-induced apoptosis of human neuroblastoma and HeLa cells (17). Caspase-4 have been reported to be physically associated with GRP78 and down-regulation of GRP78 plays a role in facilitating the activation of caspase-4 and apoptosis (18). The GRP promoters contain multiple copies of ER stress response elements. It has been previously observed that a protein complex exhibits enhanced binding to the ER stress response element of the GRP78 promoter on thapsigargin-induced stress (19, 20). Later this protein binding within the ER stress response elements. It has been previously observed that a protein complex 

EXPERIMENTAL PROCEDURES

Cell Lines and Mice—The human mammary epithelial carcinoma cells (MDAMB-231 and MDAMB-468; maintained in complete DMEM) were obtained from NCCS, India. Male Swiss albino mice were obtained from Chittaranjan National Cancer Research Institute, Kolkata, India. The SCID mice experiments were performed in NCCS, Pune. All ethical guidelines of the animal ethics committee of the Institute for handling and performing of the experiments were followed.

Synthesis and Usage of Nifetepimine—Nifetepimine has been synthesized using 3-nitrobenzaldehyde, ethyl acetocetate, and urea as described (26). For in vitro studies, 50 μM nifetepimine was used and for in vivo studies, 10 mg/kg body weight (EAC bearing mice) and 15 mg/kg body weight (SCID mice) were used.

Treatment of Animals—Male Swiss albino mice (20 g) were randomly divided into four groups of 10 animals each including (i) untreated set (non-tumor bearing), (ii) EAC bearing set (which were intraperitoneally injected with 1 × 10⁶ exponentially grown EACs in 0.25 ml sterile PBS), (iii) nifetepimine-treated set (non-tumor-bearing), and (iv) nifetepimine-treated tumor bearing set. The detailed experiments were performed using nifetepimine at a dose of 10 mg/kg body weight (injected intraperitoneally) as identified from our previous studies (26). Untreated mice received dimethyl sulfoxide instead of nifetepimine.

For the SCID mice experiments the mice were divided into two groups with 4 mice in each group (i) tumor-bearing set (injected with 2 × 10⁶ MDAMB-231 cells with Matrigel (1:1) in the mammary fat pad) and (ii) nifetepimine-treated tumor bearing set (15 mg/kg body weight injected at the site of the tumor). After 7 doses of nifetepimine injection, the mice were sacrificed and the tumors were collected for immunohistochemistry and Western blot analysis. Tumor volume was measured on every date of treatment and tumor weight was evaluated following sacrifice of the mice.

Determination of the Cytosolic Ca²⁺ Concentration—Cytosolic calcium concentration was measured using a fluorometric ratio technique. Cells were centrifuged and resuspended at a density of 10⁶ cells/ml in phosphate-buffered saline (PBS) supplemented with 1 mg/ml of bovine serum albumin and incubated in the dark with Fura-2AM (final concentration 5 μM) (Sigma) for 30 min at room temperature under slow agitation. Cells were then centrifuged and resuspended in calcium-free Hanks’ buffered saline solution (135 mM NaCl, 5.9 mM KCl, 1.2 mM MgCl₂, 11.6 mM Hepes, 11.5 mM glucose adjusted to pH 7.3 with NaOH) prior to measurements. After centrifugation, 0.5 to 1 × 10⁶ cells were suspended in 3 ml of Hanks’ buffered saline solution in a quartz cuvette and inserted into a Hitachi spectrofluorometer equipped with a stirring apparatus and a thermostatted (37 °C) cuvette holder, and connected to a PC computer. The fluorescence was recorded at 510 nm in the spectrofluorometer using an excitation source of 340 or 380 nm. Maximum Fura-2 fluorescence (Fₘₐₓ) was obtained by adding 1 μM ionomycin (Sigma) to the cell suspension in the presence of 10 mM CaCl₂, and minimum fluorescence (Fₘᵦₗ) was determined without added calcium in the presence of 5 mM EGTA (Sigma). The
cytosolic [Ca\(^{2+}\)] was calculated from the Fura-2AM fluorescence intensity as: [Ca\(^{2+}\)]_cyt = K_d (F - F_{min})/(F_{max} - F), where K_d = 224 nM for Fura-2 and R is the ratio of fluorescence values (F) (R = F340/F380).

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide Assay—The effect of nifetepimine on triple negative breast cancer cell death was assessed by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Briefly, MDAMB-231 and MDAMB-468 cells were plated and allowed to grow at 37 °C. Both cells were treated with nifetepimine and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide was added to the cells and incubated at 37 °C for 4 h. Isopropanol alcohol was added to the cells and absorbance was measured in an ELISA reader (Bio-Rad) at 570 nM.

Viability Assay: Trypan Blue Dye Exclusion Test—Viability of the cells was determined by trypan blue dye exclusion test. The cells (10 μl suspension) were placed on coverslips at the required dilutions; each coverslip was placed cell-side down onto a slide containing a drop of 0.125% trypan blue dye (w/v in sterile isotonic saline). Using gentle pressure with gauze at the coverslip edges only, excess dye solution was removed. The slides were viewed within 10 min with a light microscope at ×40 and 50 to 100 cells were counted. Viable cells remained unstained, whereas the nuclei of non-viable cells were stained blue. The ratio of viable cells to the total number of cells counted was recorded as a percent viability for each coverslip.

Calculations—Cells/ml = the number of cells per quadrant equals 10^4 cells/ml (50 cells per quadrant = 0.5 million cells/ml); total cells: cells/ml × original volume; and cell viability (%) = total viable cells (unstained)/total cells (stained and unstained) × 100 were used.

Annexin V-PI Staining—For the determination of cell death, cells were stained with propidium iodide and annexin V-FITC (BD Pharmingen) and analyzed on a flow cytometer (FACS Calibur, BD Bioscience) equipped with 488 nm argon laser light source, using Cell Quest Software (BD Biosciences). Electronic compensation of the instrument was done to exclude overlapping of the emission spectra. 10,000 total events were acquired for analysis using Cell Quest software. Annexin V/7-AAD positive cells were regarded as apoptotic cells.

Analysis of Cell Cycle—Briefly, cells were plated at a density of 1 × 10^6 cells/ml in each Petri dish in DMEM for 16–20 h. Thereafter the cells were treated with nifetepimine at 50 μM for 24 h. At the end of treatment the cells were collected and fixed with 100% methanol for 5 min at −20 °C. After centrifugation, the cell pellets were collected with 4 μg/ml of propidium iodide solution containing 100 μg/ml of RNase and 1% Triton X-100 for 30 min. Subsequently, the samples were analyzed in a FACS calibur system (BD Biosciences) using CellQuest software. The percentage of cell cycle phases was analyzed by ModFit LT software (version 2.0, BD Biosciences). Histogram display of DNA content (x axis, PI fluorescence) versus counts (y axis) has been displayed. CellQuest statistics was employed to quantitate the data at different phases of cell cycle.

Immunoblotting and Immunoprecipitation—MDAMB-231 and MDAMB-468 cells were lysed in buffer (10 mM Hepes, pH 7.9, 1.5 mM MgCl₂, 10 mM KCl, and 0.5 mM DTT) and nuclei were pelleted by a brief centrifugation. The supernatant was spun at 100,000 × g to get a cytosolic fraction. The nuclear extract was prepared in buffer containing 20 mM Hepes, pH 7.9, 25% (v/v) glycerol, 420 mM KCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.5 mM DTT, and 0.5 mM PMSF. All buffers were supplemented with protease and phosphatase inhibitor mixtures. For direct Western blot analysis, cell lysates of the particular fractions containing 30 μg of protein were separated by SDS-polyacrylamide gel electrophoresis and transferred to PVDF membrane. The protein levels of GRP78 (Sigma), cleaved caspase-9 and -3, pERK, pJAK, TFII-I, and caspase-4 was determined with specific antibodies (Santa Cruz Biotechnology). For immunoprecipitation studies GRP78 and caspase-4 immune complexes were immunoprecipitated using GRP78 antibody with Protein A-Sepharose beads (Sigma). The immunopurified were immunoblotted with caspase-4 antibody. Similarly to study the phosphorylation status of TFII-I, at first TFII-I was immune precipitated and then immunoblotted with phosphoserine and phosphothreonine antibodies (Sigma). The protein of interest was visualized by chemiluminescence. Equal protein loading was confirmed by reprobing the blots with α-actin/histone H1/GAPDH antibody (Santa Cruz Biotechnology).

Fluorescence Imaging—For the identification of subcellular localization of TFII-I under different conditions, the MDAMB-231 cells were fixed with 4% p-formaldehyde and permeabilized with Triton X-100. Cells were then stained with anti-TFII-I antibody (Santa Cruz Biotechnology) followed by FITC-conjugated secondary antibody and visualized with a confocal microscope (Carl Zeiss, Jena, Germany). For live cell imaging of calcium, MDAMB-231 and MDAMB-468 cells were treated with nifetepimine for 4 and 8 h and incubated with Fluo-3AM in Hanks’ buffered saline solution for 30 min and then visualized with a BD Bioscience Pathway microscope.

Reverse Transcriptase-PCR—GRP78 mRNA was estimated using a semiquantitative reverse transcriptase-PCR method. Briefly, total RNA from cells was extracted with TRizol reagent (Invitrogen) and reverse transcribed and amplified using the U.S. Biochemical Corp. RT-PCR kit and TaqMan DNA polymerase according to the manufacturer’s instructions by 30 cycles (each cycle consisting of 30 s at 94 °C, 2 min at 55 °C, and 2 min at 72 °C). The primers used were 5’-GGTTGGAGAGATACAGCAGAAA (sense) and 5’-GACAGCAGCTAGGGTTCACAATA (antisense).

siRNA and Transfection—MDAMB-231 and MDAMB-468 cells were transfected with 300 pmol of GRP-78-siRNA (Santa Cruz Biotechnology) and Mikk siRNA (Santa Cruz Biotechnology) and Lipofectamine 2000 (Invitrogen) separately for 12 h. Levels of the respective proteins were estimated by Western blotting.

Chromatin Immunoprecipitation (CHIP) Assay—Chromatin immunoprecipitation assay was performed using CHIP assay kit (Millipore) following the manufacturer’s instructions. Isolated chromatin was precipitated with TFII-I antibody. Input DNA, rabbit IgG-pulled DNA, served as controls for all the experiments. Immunoprecipitated DNA was then subjected to 40 cycles of PCR using primers for the respective promoter regions, 5’-CATTGTTGCGGCCTTAAGATGACCG (forward) and 5’-AGTATCGACGCGCCGTCGGC (reverse), yielding a 223-bp product. Glyceraldehyde-3-phosphate dehy-
Nifetepimine Induces Breast Cancer Cell Apoptosis

During apoptosis, a series of proteolytic cleavages of various intracellular polypeptides are initiated by the action of a unique family of serine/threonine proteases called caspases. The molecular mechanisms involving nifetepimine-induced cell death were delineated and we observed that nifetepimine induced processing of procaspase-9 and -3 in a time-dependent manner in both MDAMB-231 (Fig. 1G, left panel) and MDAMB-468 cells (Fig. 1G, right panel), thereby resulting in caspase activation. In conformity with this we also found that pre-treatment of these cells with cell-permeable inhibitors of caspase-9, caspase-3, as well as pan-caspase inhibitor followed by nifetepimine treatment significantly abrogated nifetepimine-triggered cell death clearly indicating the involvement of these caspases in the nifetepimine-induced apoptosis of MDAMB-231 (Fig. 1G) and MDAMB-468 cells (Fig. 1H).

Nifetepimine-induced Apoptosis in Breast Carcinoma Cells Involves an Overload in Cytosolic Calcium Level—As previous studies from our laboratory have already suggested that nifetepimine alters the cytosolic calcium level during T cell survival and apoptosis, we sought to check for the changes in cytosolic calcium levels during nifetepimine-mediated cancer cell apoptosis. Interestingly our findings revealed that treatment of MDAMB-231 and MDAMB-468 with nifetepimine resulted in a gradual rise in the cytosolic calcium level in a time-dependent manner ultimately creating a situation of cytosolic calcium overload on treatment for 8 h (Fig. 2A, left panel). The fluorimetric data were further reinstated from live cell fluorescence imaging, which highlighted that treatment with nifetepimine for 8 h elevated the cytosolic calcium levels significantly in both MDAMB-231 and MDAMB-468 cells (Fig. 2A, right panel). These findings thereby prompted us to further investigate the consequences of this calcium overload leading to cancer cell apoptosis.

Apoptosis

Histological and Immunohistochemistry Studies—Histological studies of liver and heart tissue of EAC bearing mice were carried out after nifetepimine administration. The MDAMB-231 tumors (control and nifetepimine treated) were excised and also subjected to histological studies using hematoxylin and eosin staining. The expression profiles of pERK were analyzed by immunofluorescence in tissue sections using anti-pERK antibody and visualized under confocal microscopy (Zeiss).

Statistical Analysis—Values are shown as mean ± S.E. except otherwise indicated. Data were analyzed and, when appropriate, significance of the differences between mean values was determined by a Student’s t test. Results were considered significant at p < 0.05.

RESULTS

Nifetepimine Induces Apoptosis in TNBC Cell Lines through Activation of the Caspase Cascade—Most of the current commercially available anti-cancer therapies induce apoptosis in various cell types, however, they are found to be mostly ineffective against the triple negative cells lacking estrogen, progesterone, and Her2/neu receptors. Our previous studies have already reported nifetepimine as a potent immune restorative agent in tumor bearers but the effect of nifetepimine on triple negative breast cancer is yet to be investigated. To reach the goal, triple negative human breast carcinoma cell lines, MDAMB-231 and MDAMB-468, were selected. Prior to application on TNBC cells we treated hormone receptor positive MCF-7 cells with nifetepimine and observed that a dose-dependent cell death occurred, producing about 45% of cell death at 50 μM (Fig. 1A, left panel). But because many commercial anti-cancer agents can induce apoptosis in MCF-7 we shifted our focus to the more resistant TNBC cells. Thus, both MDAMB-231 and MDAMB-468 cells were treated with increasing concentrations of nifetepimine for 0 to 24 h and the percentage of cell death was assessed to be about 42% at a dose of 50 μM after 24 h in MDAMB-231 cells (Fig. 1A, middle panel) and about 39% at the dose of 50 μM at 24 h in MDAMB-468 cells (Fig. 1A, right panel). Next we performed a time-dependent experiment and also observed cell death of about 40% in both MDAMB-231 and MDAMB-468 (Fig. 1B) at 24 h at a fixed concentration of 50 μM. As evident from our previous studies as well as our present findings treatment with 50 μM nifetepimine for 24 h was the optimum dose and time (Fig. 1C, left panel) because higher doses resulted in death of peripheral blood mononuclear cells thereby restricting us to use a dose of 50 μM for further studies. We also found that doses beyond 50 μM also generated toxicity in MCF-10A cells (Fig. 1C, right panel) and hence a 50 μM dose was selected for all further experiments. MCF-10A, MDAMB-231, and MDAMB-468 cell death was further confirmed using trypan blue exclusion assay (Fig. 1D). Nifetepimine was also found to increase the MDAMB-231 and MDAMB-468 cell population in the sub G0/G1 phase (Fig. 1E). The percentage of cell apoptosis was further reinstated to about 40% in both MDAMB-231 and MDAMB-468 cells (Fig. 1F) using Annexin V-PI staining.

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Elevation in the Cytosolic Calcium Level by Nifetepimine Resists in the Development of ER Stress—Calcium acts as a dynamic oscillatory molecule and thus both overload and depletion of calcium from the ER results in disruption of the ER signaling pathways thereby leading to the development of a condition referred to as ER stress (27). As our findings already depict that nifetepimine treatment for 8 h developed a situation of cytosolic calcium overload we further elucidated the chances of development of ER stress on nifetepimine treatment. Interestingly, Western blot and RT-PCR analysis identified up-regulation of the ER chaperone, GRP78 in the apoptotic cascade, MDAMB-231 and MDAMB-468 cells (Fig. 2B, left panel). As previous available ER stress inducing agent. Interestingly, our findings highlighted that treatment with thapsigargin resulted in a sustained elevation of GRP78 at both protein and mRNA levels for a period of 24 h in MDAMB-231 (Fig. 2C, left panel) and
MDAMB-468 cells (Fig. 2C, right panel). The percentage of cell death was also correlated with enhancement in the GRP78 expression levels. Interestingly, our observation revealed that the percentage of cell death remained low for both cell lines despite the elevated GRP78 levels on treatment with thapsigargin (Fig. 2D). Although doses beyond 1 μM thapsigargin
induced significant death in both cell lines, however, it produced a substantially high percentage of cell death in the peripheral blood mononuclear cells thereby restricting the dose to 1 μM (Fig. 2D). These findings are therefore indicative of the fact that down-regulation of GRP78 is essential for an enhanced percent of cell death as produced by nifetepimine on 24 h of treatment. To further validate our findings, endogenous GRP78 was knocked down in MDAMB-231 and MDAMB-468 cells and the percentage of cell death was assessed on exposure to nifetepimine or thapsigargin. Interestingly, we observed that treatment of GRP78 silenced cells with thapsigargin significantly enhanced the percentage of cell death in both cell lines (Fig. 2E) close to that produced by nifetepimine alone, thereby indicating the importance of GRP78 down-regulation in the apoptotic cascade.

GRP78-mediated Enhanced Cell Death Is Executed through Caspase 4 in MDAMB-231 Cells—As our findings discussed above clearly highlighted the importance of GRP78 as a prerequisite for enhanced cell death, we next explored the primary mediator that actually executed this cell death. It has been acknowledged that GRP78 is strongly associated with caspase-4 in the endoplasmic reticulum and this caspase-4 acts as a strong determinant in regulating ER stress-mediated cell death in different types of cancer like melanoma and colon cancers (18, 28). Thus we were prompted to investigate the role of caspase-4 in nifetepimine-mediated cell death in TNBC cells. Interestingly, our findings revealed that down-regulation of GRP78 by nifetepimine enhanced the expression of cleaved caspase-4 in MDAMB-231 cells (Fig. 2F). In fact knockdown of GRP78 using GRP78 siRNA also elevated the expression of cleaved caspase-4 on treatment with thapsigargin (Fig. 2F) thereby leading to an enhanced cell death as observed (Fig. 2E). Next, we checked the role of caspase-4 in mediating the cell death process. To do so we pre-treated MDAMB-231 cells with the caspase-4 inhibitor and then with nifetepimine. Interestingly our findings revealed that nifetepimine failed to produce activation of caspase-9 and -3 in the presence of the caspase-4 inhibitor (Fig. 2F, right panel) thereby depicting the importance of caspase-4 as the apical caspase controlling GRP78-mediated cell death process.

To further restate the importance of caspase-4 in GRP78-mediated cell death, we immunoprecipitated GRP78 with caspase-4 in MDAMB-231 cells. Our findings interestingly revealed that GRP78 was physically associated with caspase-4 in MDAMB-231 cells (Fig. 2G, left panel). However, treatment with nifetepimine declined this association and sets caspase-4 free (Fig. 2G, left panel) to activate its downstream effectors thereby leading to an enhanced percentage of cell death (Fig. 2G, right panel). Next, to re-establish the importance of caspase-4 in the ER stress-mediated cell death, we overexpressed GRP78 using GRP78 cDNA (obtained as a kind gift from Prof. Ron Prywes, Columbia University, New York) in MDAMB-231 cells. Overexpression of GRP78 strengthened its association with caspase-4 even on treatment with nifetepimine (Fig. 2G, left panel) and the percentage of cell death also remained substantially low (Fig. 2G, right panel) thereby once again highlighting the importance of caspase-4 as the primary executor in the GRP78-mediated apoptosis of TNBC cells. These results also raise the possibility of cross-talk between GRP78 down-regulation and some other signaling pathway in nifetepimine-mediated apoptosis in triple negative breast cancer cells.

MEK/ERK Down-regulation by Nifetepimine Sensitizes Breast Carcinoma Cells to ER Stress-induced Apoptosis—To evaluate the detailed mechanism of nifetepimine-induced cancer cell death, we were prompted to examine whether MEK/ERK signaling could sensitize breast carcinoma cells to ER stress-induced apoptosis. It has been acknowledged that the MEK/ERK pathway is frequently activated in breast cancer. The pathway has different effects on growth, prevention of apoptosis, cell cycle arrest, and induction of drug resistance in cell lines of various lineage (29). For all of the above reasons, MEK/ERK is an important pathway to target for therapeutic intervention. Interestingly, our results from Western blot analysis depicted down-regulation of pERK expression in MDAMB-231 and MDAMB-468 cells on nifetepimine treatment leaving total ERK expression unaffected (Fig. 3A). Our findings were further supported by confocal microscopic analysis, which confirmed the repression of pERK levels in both cell lines upon nifetepimine treatment (Fig. 3B).

To further establish the importance of pERK as a critical molecular determinant of nifetepimine-induced death of triple negative breast cancer cells, we treated MDAMB-231 and MDAMB-468 cells for 1 h with the MEK inhibitor U0126 followed by incubation with thapsigargin for 24 h. Results shown in Fig. 3C, left panel, demonstrates that U0126-mediated repression of pERK levels sensitized the MDAMB-231 and MDAMB-468 cells to thapsigargin-induced apoptosis. This was clearly evident from activation of the caspase cascade in both the cell lines on treatment with U0126 and thapsigargin (Fig. 3C, left panel). In fact the percentage of cell apoptosis was prominently enhanced in MDAMB-231 and MDAMB-468 cells (Fig. 3C, right panel) and was close to that produced by

**FIGURE 1. Nifetepimine-induced apoptosis in triple negative breast carcinoma cells via a caspase-dependent pathway.** A, graphical representation of the percentage of cell death in MCF-7 (left panel), MDAMB-231 (middle panel), and MDAMB-468 cells (right panel) induced by nifetepimine in a dose-dependent manner for 24 h. B, in a parallel set of experiment, the percentage of cell death induced by 50 μM nifetepimine was scored for time periods of 0, 4, 8, 12, 24, and 48 h in both MDAMB-231 and MDAMB-468 cells. C, graphical representation of peripheral blood mononuclear cell (PBMC) (left panel) and MCF-10A cell (right panel) death in the presence of different doses of nifetepimine. D, graphical representation of the percentage of cell death in MCF-10A, MDAMB-231, and MDAMB-468 cells induced by nifetepimine as determined by a trypan blue exclusion assay. E, MDA-MB-231 and MDAMB-468 cells were treated with 50 μM nifetepimine for 24 h and cell cycle phase distribution of nuclear DNA was determined by FACS. Histogram display of DNA content (x axis, PI fluorescence) versus counts (y axis) has been shown. F, in a double label system, unfixed MDA-MB-231 and MDAMB-468 cells were labeled with PI and annexin V-FITC and analyzed on a flow cytometer. Dual parameter dot plot of FITC fluorescence (x axis) versus PI fluorescence (y axis) has been shown in logarithmic fluorescence intensity. Annexin V/PI positivity was regarded as apoptotic cells. G, MDAMB-231 (left panel) and MDAMB-468 (right panel) cells were treated with nifetepimine for different time periods and activation of caspase-9 and caspase-3 was determined by Western blotting. H, caspase-9, caspase-3, and pan-caspase inhibitor pre-treated MDAMB-231 and MDAMB-468 cells were incubated with or without nifetepimine for 24 h and percent apoptosis was scored by annexin V/PI positivity. α-Actin was used as internal loading control. Values are mean ± S.E. of three independent experiments in each case or representative of a typical experiment: *, p < 0.05; **, p < 0.01; ***, p < 0.001.
nifetepimine alone. The MEK inhibitor alone, however, did not cause appreciable apoptotic cell death in either of these two cell lines.

To further justify the involvement of the MEK/ERK signaling in ER stress-mediated apoptosis of breast cancer cells, we knocked down MEK using MEK siRNA in MDA-MB-231 and MDA-MB-468 cells. Down-regulation of MEK expression using MEK siRNA resulted in significant caspase activation in the presence of thapsigargin in both cell lines (Fig. 3D, left panel). A marked increase in apoptotic cell death induced by thapsigargin was also observed in MEK siRNA-transfected cells (Fig. 3D, right panel). These findings cumulatively hint at the simultaneous involvement of ER stress and MEK inhibition in nifetepimine-mediated death of triple negative breast cancer cells.

Nifetepimine-mediated ERK Down-regulation Inhibits the Recruitment of TFII-I to the GRP78 Promoter—Our observations so far delineate that nifetepimine-mediated MEK/ERK inhibition resulted in down-regulation of GRP78, which is essential for producing a higher percentage of cell death in triple negative breast carcinoma cells. So here we raised the question, how does down-regulation of MEK/ERK expression regu-
late GRP78 expression in breast carcinoma cells. The GRP78 promoter contains multiple copies of the ER stress response element, which is the primary regulatory element for ER stress-mediated transcription-regulated synthesis of GRP78 (21, 22). Under conditions of ER stress, several transcription factors including transcription factor TFII-I interact with ER stress response elements of the GRP78 promoter and cause transcriptional up-regulation of its synthesis (23, 30–32). So we investigated the possible transcriptional regulation of GRP78 by TFII-I in MDAMB-231 cells in the presence and absence of nifetepimine. Interestingly, we observed that nuclear translocation of TFII-I was declined on treatment with nifetepimine for 24 h (Fig. 4A, right panel), whereas the nuclear expression level of TFII-I was found to be consistently high in the thapsigargin treated sets (Fig. 4A, left panel). The data obtained from Western blot analysis was also validated from our immunofluorescence studies that nifetepimine treatment for 24 h significantly abrogates nuclear translocation of TFII-I in comparison to that of thapsigargin (Fig. 4B, left panel) correlating our previous findings that nifetepimine down-regulates pERK expression in both the cell lines on 24 h treatment.

To check regulation of GRP78 gene transcription by nifetepimine and study the recruitment of TFII-I to the GRP78 promoter we performed a chromatin immunoprecipitation assay. The results of the CHIP assay clearly highlight that TFII-I was highly recruited to the GRP78 promoter on nifetepimine treatment for 8 h (Fig. 4C) but gradually declined on 24 h treatment in the MDAMB-231 cell line (Fig. 4C). These findings clearly state that nifetepimine-mediated pERK inhibition clearly interferes with binding of TFII-I to the GRP78 promoter, which significantly declines the GRP78 expression level, and which is a pre-requisite to execute a significant percentage of cell death in the triple negative breast carcinoma cells.

**JAK2 in Coordination with ERK Also Plays a Vital Role in Promoter Recruitment of TFII-I**—Once we elucidated the role of ERK in TFII-I nuclear translocation, we raised the question as to the involvement of any other factor in regulating nuclear translocation of TFII-I. Reports suggest that JAK2 along with ERK in TFII-I nuclear translocation, we raised the question as to the involvement of any other factor in regulating nuclear translocation of TFII-I. JAK2 along with ERK plays a vital role in regulating the nuclear translocation of TFII-I. JAK2 phosphorylates TFII-I at tyrosine 248, which facilitates its phosphorylation by ERK at 627 and 633 residues thereby enhancing its promoter binding activity (33). Thus we were prompted to investigate changes in the phospho-JAK2 expression pattern of MDAMB-231 cells in a time-dependent manner on treatment with nifetepimine. Interestingly our findings revealed that nifetepimine treatment resulted in a rise in the cytosolic calcium level, which was also associated with enhancement in pJAK2 levels from 8 h (Fig. 4D, upper panel). At the 8-h time point it was observed that pJAK2 was significantly up-regulated and pERK levels were already elevated, which facilitated nuclear translocation of TFII-I. In fact our immunoprecipitation studies also clearly highlighted that TFII-I was phosphorylated at its tyrosine and serine residues at 8 h of nifetepimine treatment (Fig. 4D, lower panel), which favored nuclear translocation and promoter recruitment of TFII-I. However at 16 h, due to a decline in the pERK levels the serine phosphorylation of TFII-I was gradually decreased and nuclear translocation of TFII-I was also retarded.

Furthermore, we also evaluated changes in the nuclear translocation of TFII-I in the presence of thapsigargin and U0126. Our observations revealed that thapsigargin alone enhanced the nuclear translocation of TFII-I in MDAMB-231 cells, whereas treatment with U0126 and transfection with MEK siRNA significantly declined nuclear translocation of TFII-I even in the presence of thapsigargin (Fig. 4E, upper panel). In fact U0126 retarded the nuclear translocation of TFII-I from 4 h of treatment (Fig. 4E, lower panel). Thus a coordinated action of JAK and ERK cumulatively regulates nuclear translocation of TFII-I, which in turn controls GRP78 expression in TNBC cells.

Thus from our in vitro findings, one can reasonably conclude that nifetepimine couples two distinct signaling pathways to induce significant killing of human triple negative breast carcinoma cells. These findings thereby prompted us to further investigate the role of nifetepimine in regressing cancer in different animal models.

**Nifetepimine Effectively Induces Apoptosis in Asects Carcinoma-bearing Swiss Albino Mice Models**—To further establish the importance of nifetepimine as an anti-carcinogenic agent we injected Swiss albino mice with $1 \times 10^6$ EAC and treated them with different doses of nifetepimine. Nifetepimine significantly retarded tumor progression (Fig. 5A) in a dose-dependent manner (5, 10, and 20 mg/kg body weight) as was evident from the decline in the EAC cell number collected from the Swiss albino mice after nifetepimine treatment for 6 weeks (Fig. 5B). We also observed that nifetepimine was most prominently effective at a dose of 10 mg/kg body weight as the decrease in the splenic mononuclear cell number produced by the growing tumor was restored close to normal levels on
treatment with 10 mg/kg of nifetepimine (Fig. 5B). The EAC volume was also found to decline significantly on treatment with 10 mg/kg of nifetepimine for 6 weeks (Fig. 5C). The percentage of EAC cell death produced on nifetepimine treatment was confirmed to about 40% from annexin V staining (Fig. 5D). Histopathological data also supported our conclusions showing that nifetepimine delivered complete protection from the damage produced by the tumor to the kidney and liver (Fig. 5E). Further studies also showed that the tumor burden resulted in a significant elevation in the serum glutamate pyruvate transferase, serum glutamate oxaloacetate transaminase, and alkaline phosphatase levels. However, nifetepimine provided a complete safeguard to the liver restoring the levels to normal (Fig. 5F). Our Western blot analysis also revealed down-modulation in the pERK levels as well as activation of the caspase cascade, which is also in line with the in vitro findings (Fig. 5G). Thus our findings so far suggest that nifetepimine acts as a potent anti-carcinogenic agent in both in vitro as well as in vivo models.

Nifetepimine Attenuates in Vivo Breast Tumor Growth in SCID Mice Xenograft Models—The in vitro experimental analysis so far thereby prompted us to investigate the role of nifetepimine on in vivo breast tumor regression. Accordingly, MDAMB-231 cells were injected in the mammary fat pad of SCID mice and after tumor development, were injected with
nifetepimine at doses of 10, 15, and 25 mg/kg body weight. Interestingly, we observed a slowed tumor growth in a dose-dependent manner for a period of 4 weeks (Fig. 6A, left and right panel). In reference to the above findings, another subsequent study using a single dose (15 mg/kg body weight) of nifetepimine for 6 weeks was carried out. Interestingly nifetepimine was found to significantly slow down tumor growth in SCID mice models (Fig. 6B, I and II). Our findings clearly elucidated that nifetepimine subsequently attenuated both tumor weight (Fig. 6C) and volume (Fig. 6D) in MDAMB-231 tumor bearing SCID mice. The tumor sections were also analyzed histopathologically using H&E staining. The control tumors exhibited higher infiltration, poorly differentiated structures, and enhanced nuclear polymorphism in comparison to those treated with nifetepimine (Fig. 6E). The tumor sections were also analyzed immunohistochemically using pERK antibodies. A prominent down-regulation in pERK levels was observed in the sets treated with nifetepimine (Fig. 6F). Nifetepimine-induced apoptosis was also finally confirmed by Western blot analysis using anti-caspase antibodies (Fig. 6G). In fact we also observed retardation in nuclear translocation of TFII-I in the sets treated with nifetepimine (Fig. 6G). Thus cumulatively our findings illustrated that nifetepimine apart from being a potent immune-restorative agent also acts as an effective anti-cancer agent against triple negative breast cancer cells in both in vitro and in vivo systems.

**DISCUSSION**

Increased apoptosis resistance and recurrence of tumors are the major roadblocks to effective treatment of TNBC (34–36) thereby posing a significant clinical challenge to the researchers worldwide. Therefore by elucidating the critical molecular

**FIGURE 4.** Nifetepimine-mediated ERK down-regulation inhibits the recruitment of TFII-I to the GRP78 promoter in MDAMB-231 cells. A, the cytosolic and nuclear expression levels of TFII-I in MDAMB-231 cells treated with thapsigargin (Tg) (left panel) and nifetepimine (right panel) at different time periods as determined from Western blot analysis. B, left panel, nuclear translocation of TFII-I on treatment with thapsigargin and nifetepimine in MDAMB-231 cells was determined using fluorescence microscopy. Right panel, the Pearson’s and Manders coefficients determining the level of overlap of DAPI and FITC on treatment with thapsigargin (Tg) and nifetepimine has been represented graphically. C, schematic representation of the GRP78 promoter and sequence of the forward and reverse primer used for CHIP analysis. CHIP assay with the nifetepimine-treated MDAMB-231 cell at different time periods determining the recruitment of TFII-I to the GRP78 promoter. D, changes in the expression levels of pJAK2 in MDAMB-231 cells treated with nifetepimine at 0, 8, 16, and 24 h (upper panel); MDAMB-231 cells were treated with nifetepimine for 0, 8, 16, and 24 h and immunoprecipitated (IP) with TFII-I antibody and then immunoblotted with TFII-I, phosphoserine, and phosphotyrosine antibodies (lower panel). E, the cytosolic and nuclear expression levels of TFII-I in MDAMB-231 cells treated with thapsigargin, thapsigargin + U0126, and thapsigargin + MEK siRNA as determined by Western blotting (upper panel). Changes in the nuclear expression pattern of TFII-I on treatment with U0126 at 0, 8, 16, and 24 h are indicated (lower panel). α-Actin, histone H1, and GAPDH were used as internal loading controls.
mechanisms of TNBC and targeting these mechanisms with alternative therapeutic agents may help to make significant clinical strides in the coming years.

Various mechanisms have been implicated in the induction of apoptosis by ER stress (37–40). However, none of them seemed to be significantly operative in TNBC cells thereby rendering them significantly resistant to apoptosis induced by the ER stress inducer, thapsigargin. Nevertheless, ER stress-induced apoptosis was found to be readily triggered in TNBC cells via a caspase-dependent mechanism.

FIGURE 5. Nifetepimine regresses EAC growth in Swiss albino mice models. A. Swiss albino mice were injected intraperitoneally with EAC and then subjected to 10 mg/kg of nifetepimine treatment for 6 weeks. Representative mice from control, EAC bearing, and nifetepimine-treated EAC bearing sets were photographed after 6 weeks. B, tabular representation showing the changes in EAC number and splenic mononuclear cell number on treatment with different doses of nifetepimine for 6 weeks. C, graphical representation of the changes in the EAC volume of nifetepimine-treated and untreated mice for a period of 6 weeks. D, the percentage of cell apoptosis as determined from annexin V/PI staining in nifetepimine-treated and untreated EAC bearing mice models after sacrifice at the end of 6 weeks. E, histological sections of liver and kidney from the animals were stained with hematoxylin and counterstained with eosin and microscopically analyzed for histopathological examinations of tissue toxicity like cellular damage and vacuolization. F, blood was collected after sacrifice from the mice of the above sets and the serum levels of SGPT, SGOT, and ALP were measured and represented graphically. G, Western blot analysis depicting the changes in pERK, caspase-9 and -3 expression levels from EAC bearing nifetepimine-untreated and treated sets. α-Actin was used as internal loading control. Values are mean ± S.E. of three independent experiments in each case or representative of typical experiment.
when the MEK/ERK pathway was inhibited. These findings thereby suggested that the MEK/ERK pathway significantly antagonize the apoptosis inducing potential of ER stress in TNBC cells.

In accordance with the above findings in the present study we took a closer look in investigating the anti-carcinogenic property of nifetepimine, a synthetic dihydropyrimidone already known for its potent immune-restorative property in cancer...
Nifetepimine Induces Breast Cancer Cell Apoptosis

patients (26). Nifetepimine was found to be a potent inducer of apoptosis in triple negative breast cancer cells. But a clear understanding of the exact molecular targets of nifetepimine was required to finally proceed for pre-clinical trials and specifically designate the exact amount of nifetepimine required for the chemopreventive action in humans in the future. This report thus furnishes multiple evidence to confirm that nifetepimine involve two distinct signaling pathways resulting in activation of the executioner caspases, culminating to apoptosis of TNBC cells.

It is known that calcium is a ubiquitous second messenger controlling a broad range of cellular functions including growth and proliferation. Changes in the calcium homeostasis of ER initiate a cascade of quality control signaling mechanisms that restore normal ER function (41). Prolonged or severe ER stress, however, overwhelms cellular protective mechanisms, ultimately triggering cell death via a caspase-dependent mechanism. Nifetepimine perturbed the cellular calcium homeostasis in TNBC cells resulting in ER stress development to induce minimal apoptosis in these cell types. However, a decline in the expression level of the ER chaperone protein, GRP78, with an associated enhanced cell death on prolonged exposure to nifetepimine provided us the molecular clues of the involvement of other distinct signaling pathways in nifetepimine-induced TNBC cell death.

It has been acknowledged that the MEK/ERK pathway is a key intrasignaling pathway that is frequently activated in most types of cancers (29). This constitutively activated MEK/ERK pathway seems to play an important role in protection of the TNBC cells from ER stress-induced apoptosis as inhibition of MEK by either the chemical inhibitor or MEK siRNA markedly sensitized the TNBC cells to thapsigargin-induced apoptosis. Our investigation of the mechanism involved in the MEK/ERK-mediated inhibition of ER stress-induced apoptosis led to a focus on GRP78, a ER chaperone protein that is known to inhibit apoptosis by multiple mechanisms (12). Our findings clearly demonstrated that GRP78 was decreased and its up-regulation by ER stress was blocked on inhibition of MEK in the TNBC cells. In fact, knockdown of GRP78 also enhanced the death produced by thapsigargin clearly suggesting that GRP78 plays a significant role in MEK/ERK-mediated regulation of apoptotic signaling in the cells subjected to ER stress. Further evaluation of the causes behind GRP78 down-regulation and enhanced apoptosis also led to the focus that GRP78 was physically associated with caspase-4 in the MDAMB-231 cells. In fact down-regulation of GRP78 facilitated caspase-4 activation, which acted as the apical caspase in activation of caspase-9 and caspase-3. Hence forth we have concluded that nifetepimine-mediated TNBC cell death is implemented by caspase-4, which is otherwise suppressed by the ER chaperone, GRP78. Thus the coupling of ER stress and MEK/ERK inhibition by nifetepimine provides a realistic possibility for future cancer therapy particularly in TNBC cells.

To gain insight into the cross-talk between ER stress and the MEK/ERK pathway we further investigated the possible mechanistic regulation of GRP78 transcription by the MEK/ERK pathway. It has been acknowledged that chemically induced ER stress results in induction of TFII-I, which transcriptionally up-regulates GRP78 under these conditions (42). In fact other reports also suggest that knockdown of TFII-I results in abrogation of TFII-I binding to the GRP78 promoter leading to down-regulation in GRP78 expression (42). In coalesces with the above mentioned reports our observations also revealed that thapsigargin-induced ER stress resulted in transcriptional up-regulation of GRP78 by enhancing the promoter binding activity of TFII-I. However, as discussed by Kim and Cocran (23), the promoter activity of TFII-I is regulated by phosphorylation of this transcription factor by ERK. In fact TFII-I is initially phosphorylated by JAK at tyrosine 248, which thereby facilitates its phosphorylation in serines 627 and 633 by ERK, which is a major pre-requisite for its nuclear translocation and promoter binding activities (23, 33). However, it is also acknowledged that in the absence of Tyr phosphorylation by JAK, ERK is incapable to phosphorylate TFII-I at its serine residues (33). Our observations are in line with the above mentioned findings that pJAK up-regulation by nifetepimine favors tyrosine phosphorylation but pERK down-regulation after 8 h gradually declined the phosphoserine levels of TFII-I in TNBC cells, which significantly impaired nuclear translocation and promoter activity of TFII-I thereby down-regulating GRP78 expression and inducing significant apoptosis in these cell types. Thus from the above findings one can reasonably conclude that nifetepimine-mediated pERK down-modulation is a critical determinant in regulating GRP78 transcription and monitoring the level of ER stress essential for producing substantial apoptosis in TNBC cells. Our in vitro findings were also supported by our in vivo results where significant slowed tumor growth was observed in both EAC bearing Swiss albino mice models as well as in the MDAMB-231 tumor bearing SCID mice xenograft models thereby clearly highlighting the importance of nifetepimine as a potential anti-cancer agent.

In conclusion, data presented here for the first time provides direct evidence that nifetepimine couples ER stress with MEK/ERK inhibition to induce noteworthy apoptosis in triple negative breast carcinoma cells. In the present study we also delineated the detailed molecular mechanisms of GRP78 gene transcription regulation by nifetepimine and its importance in TNBC cell apoptosis. Nifetepimine, in fact acts as a double edged sword by rejuvenating the immune system (26) on one hand and regressing the tumor on the other. By this concerted action, nifetepimine might play a vital role as a therapeutic agent for treatment of triple negative breast cancer. The present findings thus cumulatively help in unraveling the undiscovered pathway of nifetepimine-induced apoptosis in triple negative breast carcinoma cells and raises new hopes for efficient therapeutic approaches for treatment of breast cancer in the future.

Acknowledgments—We thank Prof. Tamara Lah and Dr. Neža Podergajs, National Institute of Biology, Ljubljana, Slovenia, for kindly gifting the MCF 10A cell line. We are also thankful to Prof. Ron Prywes, Columbia University, New York, for gifting us the GRP78 cDNA. Thanks are due to A. Basu, R. Dutta, and K. Das for technical help. We acknowledge the Center for Research in Nanoscience and Nanotechnology, University of Calcutta, for providing some instrumental facilities.
