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2-Methoxyestradiol mediated signaling network in pancreatic cancer

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1. ABSTRACT

2-Methoxyestradiol (2-ME), an endogenous metabolite of 17 beta-estradiol, is known to be a potent inhibitor of neovascularization. Our previous studies have shown that 2-ME can suppress growth of pancreatic tumor cells *in vitro* and *in vivo* by the induction of apoptosis (Cancer Res 66: 4309-18, 2006). In order to better comprehend the signaling modulators of 2-ME in pancreatic cancer, we employed a PowerBlot Western Array screening system. Our proteomic profiling has provided framework to define the novel mechanisms of actions of 2-ME in pancreatic cancer. Interestingly, this high-throughput analysis identified proteins such as Rac1, Gelsolin, Glucocorticoid receptor (GR), Smad 2/3, Smad 4, IRS-1, which were not previously reported with 2-ME response. Interestingly, 2-ME modulated down regulation of GR level is accompanied by NF-κB activation in 2-ME responsive but not in resistant pancreatic cancer cells. In view of this observation, possible reciprocal relationship between GR and NF-kappaB activation might be an important regulatory factor in 2-ME mediated demise of a subpopulation of pancreatic cancer cells.

2. INTRODUCTION

2-Methoxyestradiol (2-ME) is a promising antiangiogenic and anticancer agent (1-12). The anti-proliferative activity of 2-ME in various tumor cell lines arises mainly from triggering of apoptosis. In contrast to actively proliferating cells, quiescent cells are not responsive to cytotoxic effects of 2-ME. Despite being an estrogen metabolite, the anti-proliferative activity of 2-ME is independent of the presence of estrogen receptor (10). In addition to its anti-proliferative effect, 2-ME has been found to regulate various steps in the angiogenesis cascade such as migration, invasion, collagen matrix and tubule formation (8, 11). Several studies provide considerable evidence for the anti-tumor ability of 2-ME. Besides primary tumors, 2-ME also can effectively control metastatic invasion of cancer (3, 13). The postulated mechanisms of action of 2-ME comprise of i) inhibition of endothelial cell proliferation and migration, ii) the down regulation of the expression of vascular endothelial growth factor (VEGF) or Hypoxia inducible factor (11) and iii) induction of endothelial cell apoptosis (14).

Apoptosis mainly occurs through two types of pathways (15-17): the death receptor pathway (extrinsic) and the mitochondrial pathway (intrinsic). Interestingly, previous observations (1, 2) from our laboratory demonstrate that 2-ME up regulates Fas (a member of cell membrane localized death receptor) as well as can perturb mitochondrial membrane in pancreatic cancer cells. This implicates 2-ME in triggering apoptosis in pancreatic cancer cells by converging extrinsic and mitochondrial intrinsic pathways (1, 2). Despite our investigation registers the hierarchy of molecular events associated with 2-ME mediated pancreatic cancer cell death, the molecular mechanism by which 2-ME can alter the expression of Fas in MIA PaCa-2 cells is obscure. Like 2-ME, multiple reports have documented the induction of Fas in response to such stimuli as viral infection, hypoxia and chemotherapeutic agents (18-21). Previous studies have suggested that NF-κB can act as an inhibitor of apoptosis induced by TNF (22, 23) but not by Fas (24, 25). Furthermore, 5' flanking region of the human Fas promoter contains NF-κB consensus motifs (24, 25). Since 2-ME altered the expression of Fas, we sought to determine transcriptional activation status of NF-κB in relation to 2-ME induced pancreatic cancer cell death**.** In pursuant to these studies, we have observed that 2-ME can enhance NF-κB activation in a subpopulation of pancreatic cancer cells with concomitant apoptosis. While NF-κB is constitutively activated in numerous tumors (26, 27), this tumor microenvironmental molecule also plays an antimalignancy role in various neoplasms (28-31). Similar to other microenvironmental molecules such as TGF-β or TNFα (32), NF-κB also bears dual functions by playing opposing antiapoptotic and proapoptotic roles depending on the cell type (33-38).

In the present study we also employed high-throughput western array (39, 40) to identify alterations of specific proteins in pancreatic carcinoma cells under 2-ME stress. One interesting observation we noted that 2-ME can attenuate GR level in 2-ME exposed MIA PaCa-2 cells. The previous reports in the literature (41-43) documented mutual antagonism between GR and NF-κB. Thus the status of enhanced NF-κB transcriptional activity in 2-ME exposed pancreatic cancer cells might be reciprocally regulated by GR level**.**

3. MATERIALS AND METHODS

3.1. Cell lines

Human pancreatic cancer cells MIA PaCa-2 (carcinoma), PANC-1 (ductal adenocarcinoma) and Hs 766T (metastatic) were grown in RPMI supplemented with 10% fetal bovine serum and 50 μg/mL gentamicin at 37°C in a 5% CO_2 humidified atmosphere.

3.2. Sample preparation for powerblot analysis

Cells were grown in 10-cm diameter tissue culture plates and treated with 5 μM 2-Methoxyestradiol (2-ME) for a period of 24 hrs. Following treatment with 2-ME, the media were removed by aspiration and were rinsed with PBS. Cells were lysed in a boiling buffer containing 10 mM Tris, pH 7.4, 1 mM sodium orthovanadate and 1% SDS. The cell lysates were collected and briefly heated in a microwave oven. Next, lysates were subjected to sonication for 15 s to shear genomic DNA followed by determination of protein concentration by using BCA reagent (Pierce Chemical Co, Rockford, IL). The samples were frozen at -80°C.

3.3. PowerBlot Western array analysis

Using Cancer Template 1 and 2 antibodies, the differential expression of proteins in DMSO control and 2-ME treated MIA PaCa-2 cells were determined by high-throughput Western array by BD Biosciences /Pharmingen (San Diego, CA). Lysates containing 200 μg of protein were loaded on a big well of a 4-15% SDS polyacrylamide slab gel (13X10 cm). The gel was run for 1.5 hrs at 150 volts. The proteins were transferred on to Immobilon-P nylon membrane (Millipore) for 2 hrs. After transfer the membrane was blocked in a blocking buffer containing 5% milk. Next the membrane was clamped with a western blotting manifold that isolates 42 channels across the membrane. In each channel, a complex antibody cocktail was added and allowed to hybridize for one hour at 37º C. The blot was removed from the manifold, washed and hybridized for 30 minutes at 37ºC with secondary goat anti-mouse conjugated to Alexa680 fluorescent dye (Molecular Probes).The membrane was washed, dried and scanned at 700nm (for monoclonal antibody target detection) using the Odyssey Infrared Imaging System (LI-COR).

Data analysis includes raw and normalized signal intensity data from each blot with changes greater than 1.25 fold. All blots were normalized to the sum intensity of all valid spots on a blot then multiplied by 1,000,000. The Normalized Quantity for experimental bands expressed as a ratio of the Normalized Quantity for the corresponding control bands. The ratio was used to determine increases or decreases in protein expression. Samples were run in triplicate and analyzed using a 3X3 matrix comparison method. For example, run 1 of control was compared to runs 1, 2, and 3 of experimental. Run 2 of control was compared to runs 1, 2, and 3 of experimental. Run 3 of control was compared to runs 1, 2, and 3 of experimental. Results are expressed as fold change, a semi-quantitative value that represents the general trend of protein changes, either increasing or decreasing, for the experimental sample relative to control. The changes greater than 1.25 fold in at least 8 out of 9 comparisons are presented here.

3.4. Conventional Western blot analysis

Cells were lysed in ice cold buffer containing 50 mM Tris-HCl (pH 7.4) , 0.25 mM NaCl, 1% NP-40, 0.1% SDS, 0.5% Deoxycholate, 1mM EDTA, 1mM sodium orthovanadate and protease inhibitor cocktail (Roche). Proteins were electrophoresed through a SDS-polyacrylamide gel and transferred onto nitrocellulose membrane (Millipore) by electroblotting. The membranes were incubated in blocking buffer (5% nonfat dried milk, 10 mM Tris (pH 7.5), 100 mM NaCl and 0.1% Tween 20). Immunoblotting for protein expression was performed using antibodies (BD Biosciences/Transduction Laboratories) against selected proteins, the levels of which were altered as detected by the

PowerBlot analysis. The blots were developed using the enhanced chemiluminescence system (Amersham).

3.5. Cell based ELISA

Cells were seeded into 96 well plate. Both control and 2-ME treated cells were fixed with 4% buffered formalin followed by sequential incubation with IκB-α Primary and HRP-conjugated Secondary antibodies. The amount of bound antibody in each well was colorimetrically (OD 450 nm) determined using a developing solution (Superarray Bioscience Corp). The absorbance readings were then normalized to relative cell number as determined by cell staining solution (OD595). The following controls were used: i) Blank wells (not seeded with any cells) ii) Detection control wells (seeded with cells and incubated with secondary antibody only).

3.6. NF-κB transcription factor assay

Nuclear proteins were extracted using NE-PER reagents (Pierce Biotechnology) from control and 2-ME treated MIA PaCa-2 cells. DNA binding activity of NF-κB was measured by employing the non-radioactive assay kit in 96 well format (Upstate Biotechnology). The biotinylated oligo containing the flanked DNA binding consensus sequence for NF-κB (5'-GGGACTTTCC-3') was mixed with nuclear extract in the Transcription Factor Assay (TFA) Buffer (Upstate).The active NF-κB protein was immobilized on the biotinylated double stranded oligonucleotide capture probe bound to the streptavidin coated plate well. The bound NF-κB was detected with rabbit anti- NF-κB p65 primary antibody and a highly sensitive HRP-conjugated secondary antibody. The colorimetric detection was carried out using a spectrophotometric plate reader (μQuant, Biotex Instruments, Inc).

3.7. Apoptosis assay

Control and 2-ME exposed cells were either processed for chromatin condensation analysis by DNA group binding dye, 4', 6'- diamidino -2-phenylindole (DAPI) fluorescence (1,2, 44-46) or poly-ADP ribose polymerase (PARP) cleavage analysis (1,2, 44-46) by immunoblotting with monoclonal antibody against PARP (BD Biosciences, CA). For DAPI staining, cells were washed and fixed followed by mounting in a fluid containing 2 μg/ml DAPI (Vector Laboratories, CA). A Nikon Eclipse E600 Fluorescence microscope was used to visualize nuclear stain (1, 2).

4. RESULTS

4.1. Differential expression of proteins regulated by 2-ME in pancreatic carcinoma cells

Extracts from MIA PaCa-2 cells, grown for 24 hrs in control medium or in medium supplemented with 5 μM 2-ME, were subjected to high-throughput Western array (Figures IA-ID) using Cancer Template 1 & 2 series of antibodies (BD Biosciences). When control blots are compared with the corresponding blots derived from 2-ME treated cells, we noted alterations in several proteins associated with apoptosis signaling pathway, cell cycle, DNA repair, hypoxia and other fundamental cellular processes (Table 1). A representative blot of proteins extracted from untreated cells is shown in Figure 1A & 1C. The corresponding blots derived from 2-ME exposed cells demonstrating changes (increases or decreases) in some proteins such as GR , Gelsolin, Rac1, Cdk1, Cdk2 , IRS-1 (enclosed within a box) are presented in Figures 1B & D.

Conventional western blots were performed to confirm the Western array data for selected proteins (Figure 2). The analysis of the antibody array data uphold the previous observation that 2-ME diminishes the level of cysteine proteases such as procaspase 8, procaspase 3 and another proapoptotic member of Bcl-2 family protein Bid (Table 1). The cleavage of these proteins is essential for the induction of cell death in 2-ME exposed pancreatic cancer cells (1, 2). PowerBlot data also indicated the elevated level of Jun, an AP-1 family transcription factor in MIA PaCa-2 cells due to 2-ME exposure. Interestingly, we have previously reported the significant activation of Jun kinase when we assessed the status of JNK activity by immunocomplex kinase assay in untreated and 2-ME exposed MIA PaCa-2 cells (2). This data conforms to previous report (47) demonstrating NGF induced activation of the Jun kinase pathway, increased c-Jun protein levels and c-Jun phosphorylation with concomitant apoptosis in sympathetic neurons.

4.2. 2-ME and Transforming growth factor β (TGFβ)-Smad signaling in pancreatic cancer

PowerBlot as well as conventional Western blot analysis demonstrate the diminished expression of Smad 2/3 and Smad 4 proteins in 2-ME treated pancreatic cancer cells (Figure 2A). Smads are evolutionarily conserved proteins identified as mediators of transcriptional activation by members of the TGFβ superfamily. TGFβ-Smad signaling pathway can inhibit the growth of human epithelial cells and plays a central role in tumor suppression (48, 49). Alterations that affect expression or activity of components of the TGFβ pathway can contribute to tumor progression and metastasis. The tumor suppressor gene Smad4/DPC4, a key transcription factor in transforming growth factor beta (TGF-β) signaling cascades, is inactivated in 50% of pancreatic adenocarcinomas (50). It is plausible that 2-ME mediated attenuation of inactivated Smad proteins might play an essential role in death advantage of pancreatic cancer cells.

4.3 Decreased expression of IRS-1 following 2-ME exposure

Initial high-throughput immunoblotting indicated that IRS-1 protein level was diminished in 2-ME exposed MIA PaCa-2 cells (Fig.1). This decrease in IRS-1 level was validated by conventional Western blot analysis (Figure 2A). IRS-1, a multisite docking protein, has been implicated in mitogenic signaling due to activation of the insulin receptor and the insulin like growth factor I. Previously, IRS-1 mRNA transcript was noted to be overexpressed in pancreatic cancer specimens when compared with normal pancreas suggesting the role of IRS-1 in the signaling pathway leading to excessive growth

Table 1. List of proteins found to be modulated in 2-ME exposed MIA PaCa-2 cells by PowerBlot analysis

Protein Name	Biological Function	Increase/ Decrease	Change in level
Caspase-7	Cysteine protease ; involved in apoptosis	-	> 2 fold
Caspase-8	Cysteine protease ; involved in apoptosis	-	> 2 fold
Caspase 3	Cysteine protease ; involved in apoptosis	-	> 2 fold
Bid	Bcl2 family related protein regulating apoptosis	-	> 1.5 fold
MSH3	Nucleotide mismatch repair protein	-	> 2 fold
Mre 11	DNA double-strand break repair	+	> 1.5 fold
Topo IIa	DNA Topoisomerase	+	> 2 fold
DNA Topo I	DNA Topoisomerase	-	> 1.25 fold
Hck	Tyrosine kinase; inhibition of phagocytosis	-	> 1.5 fold
Chk2	Cell-cycle-checkpoint kinase	-	> 2 fold
Cdk4	Cell cycle regulatory kinase	+	> 2 fold
Cdk2	Cdc2 related cell cycle protein kinase	+	> 2 fold
Cdk1/Cdc2	Cell cycle regulatory kinase	+	≥ 2 fold
PKB Kinase/PDK1	Serine/threonine protein kinase; role in cell proliferation	+	≥ 2 fold
ILK	Integrin linked kinase; regulates cell migration	-	≥ 2 fold
Rac1	Ras related GTPase protein	-	> 1.5 fold
P190	Tyrosine phosphorylated Ras-GTPase activating protein	-	≥ 2 fold
5-Lipooxygenase	Initial enzyme that converts arachidonic acid to leukotrienes	+	≥ 2 fold
Ufd2/E4	E4 polyubiquitylating enzyme; regulates chromosome condensation and separation during mitosis	-	> 1.5 fold
c-Cbl	E3 ubiquitin ligase	-	≥ 2 fold
Gelsolin	A member of a large family of actin-severing and -capping proteins; has been shown to inhibit apoptosis	-	> 1.25 fold
gamma-Catenin	E-Cadherin binding protein regulating adhesion	-	> 1.25 fold
Paxillin	A cytoskeletal component ; role in focal adhesion	+	> 2 fold
Jun	AP-1 family transcription factor	+	> 2 fold
Smad 2/3	Component of TGF β signaling; gene activation	-	> 1.5 fold
Smad 4	Component of TGF β signaling; gene activation/development	-	> 2 fold
HIF-1b	Hypoxia inducible factor; mediates gene transcription in response to hypoxia	-	≥ 2 fold
Glucocorticoid receptor	Transcriptional regulators of genes required for embryonic development and adult homeostasis	-	≥ 2 fold
IRS-1	Insulin receptor substrate adapter protein	-	> 2 fold
SHC	Adaptor protein in Ras pathway	+	> 1.5 fold
mEPHX	Microsomal epoxide hydrolase	+	> 1.5 fold
GAGE	Tumor associated antigen	-	≥ 2 fold

stimulation in human pancreatic cancer (51). Apparently, decreased level of IRS-1 might have some implication to apoptosis inducing ability of 2-ME in pancreatic cancer cells.

4.4. Modulation of cell cycle regulatory proteins by 2-ME

Treatment of human cancer cell line MIA PaCa-2 with 2-ME increases Cyclin B1 protein and its associated kinase Cdk1/Cdc2 (Table-1; Figure 2B). One possible mechanism of 2-ME induced Cyclin B1 overexpression could be deregulation of Cyclin B1 promoter so that there is greater initiation of the transcription. Alternatively, there might be inhibition of Cyclin B1 protein degradation that is critical for progression through mitosis (52). It is worth mentioning that Cdc2 kinase activity is significantly increased (1.7-fold, $P < 0.005$) after 2ME exposure of esophageal carcinoma cells when compared to vehicle-treated controls.

4.5. Effect of 2-ME on Hypoxia Inducible Factor /VEGF signaling

We observed here down regulation of both α and β-subunit of HIF-1 in pancreatic cancer cells following 2-ME exposure (Figure 2A & B). Several studies have shown earlier that optimal transcriptional control of the vascular endothelial growth factor (VEGF) promoter requires binding of HIF-1α (11). Human pancreatic cancer cells secrete proangiogenic molecules VEGF which is involved in tumor vascularization; a vital process for the progression of all solid tumors from a small, localized focus to an enlarging tumor with the capability to metastasize. Interestingly, 2ME is known to down regulate VEGF level in 2-ME treated MIA PaCa-2 cells (53). Thus deregulation of HIF-1 level might disrupt transcriptional activation of VEGF expression in MIA PaCa-2 cells as observed earlier in the case of breast and prostate cancer (11).

4.6. 2-ME can down regulate Glucocorticoid receptor (GR) in MIA PaCa-2 cells

Glucocorticoids exert their effects by binding to the GR, a transcription factor capable of regulating several genes in a positive or negative way (41-43). Initially, western array indicated approximately 2 fold reduction in the expression of GR in 2-ME exposed MIA PaCa-2 cells (Table 1). We further confirmed this down regulation of GR by conventional immunoblotting (Figure 2B). In addition to MIA PaCa-2, ductal adenocarcinoma cells PANC-1 also exhibit decreased level of GR in response to 2-ME (Figure 3A). On the contrary, in 2-ME insensitive metastatic pancreatic cancer cells Hs 766T, GR level remains unaltered (Figure 3B).

4.7. 2-ME increases nuclear factor-κB (NF-κB) DNA binding activity by diminishing total IκBα level in MIA PaCa-2 cells

The molecular mechanism of 2-ME induced Fas upregulation is not well understood. One possibility might be the transcriptional regulation of death receptors by NF-κB. This important molecule regulates the activities of

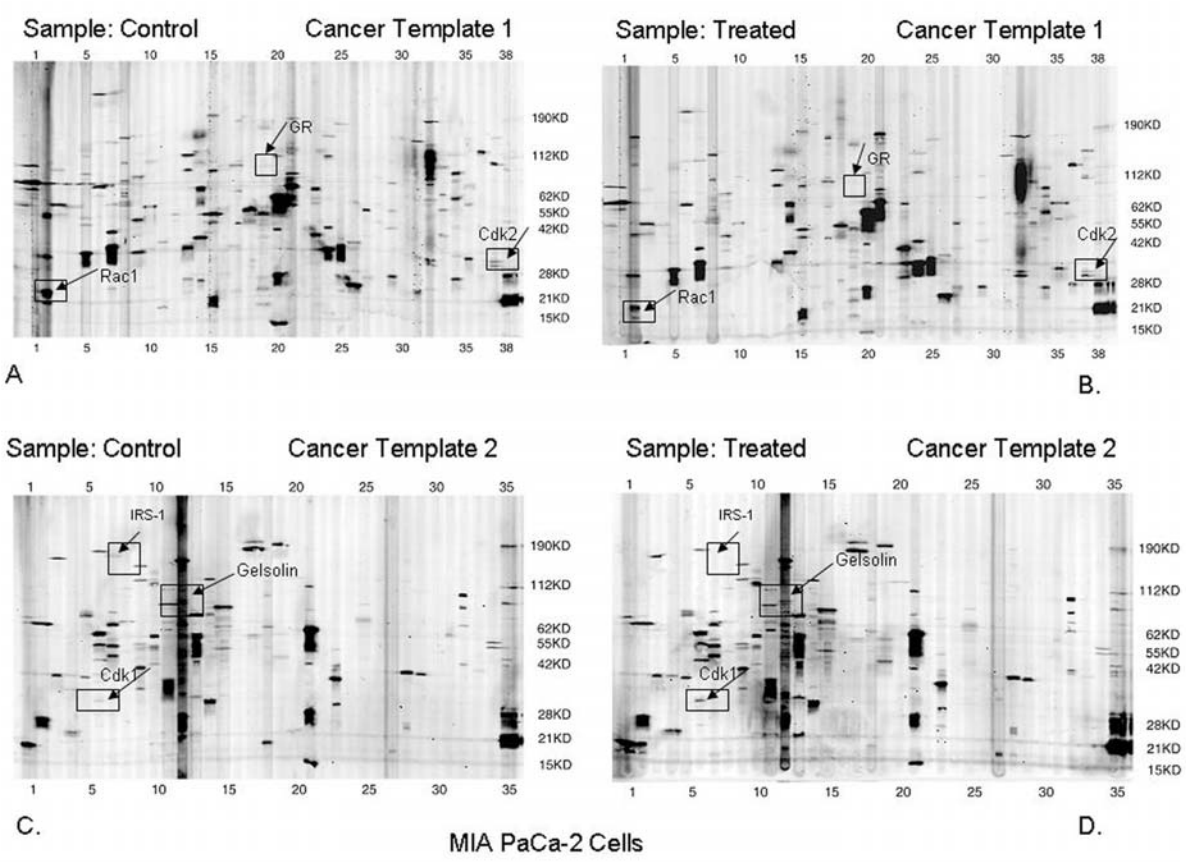

Figure 1. Analysis of the differential expression of proteins in control and 2-ME treated MIA PaCa-2 cells by high-throughput Western array. 200 μg of total cellular proteins from control (vehicle solvent DMSO treated) and 2-ME treated cells were loaded on a slab gel and PowerBlot western analysis was performed using Cancer templates 1 and 2 (BD Biosciences) as described in Materials and Methods. The numbers above the blot represent the lanes used for hybridization with a complex antibody cocktail (4-5 antibodies per lane). After hybridization with the primary antibodies the blots were probed with secondary goat anti-mouse conjugated to Alexa680 fluorescent dye. The membranes were scanned at 700 nm using the Odyssey Infrared Imaging System. Some of the proteins showing differential levels are denoted within a box on the blots.

many signaling pathways in the intracellular signaling network, thereby playing a critical role in determining cellular responses to extracellular stimuli (22, 23). Although NF-κB is mostly known to suppress programmed cell death, under certain circumstances, NF-kB activation is associated with or required for cell death (18-21, 27-37). A few proapoptotic NF-κB transcriptional target genes appear to be involved in this effect. These proapoptotic targets include the death receptors such as Fas (CD95), TRAIL receptors DR4, DR5 and DR6 (37). To explore the possibility that 2-ME induced Fas upregulation in MIA PaCa-2 cells might be mediated by NF-κB, we were primarily interested to investigate the NF-κB activity in 2-ME challenged pancreatic carcinoma cells. The ELISA-based colorimetric NF-κB functional assay demonstrates significant increase in its activation following 2-ME exposure of MIA PaCa-2 cells (Figure 4D). Moreover, NF-κB activity was indirectly monitored by detecting the amount of IκB-α (inhibitor of NF- κB) by cell based ELISA as well as immunoblotting. The ubiquitination of phosphorylated IκB-α and its subsequent degradation by the 26S proteasome (27) leads to the translocation of NF-κB to the nucleus where it activates gene transcription. Figs. 4A & C clearly demonstrate the decrease of total IκB-α protein in MIA PaCa-2 cells challenged with 2-ME for 16 hrs. It is worth mentioning that time course studies with 2-ME exposed MIA PaCa-2 cells at earlier points (2,4 & 8 hrs) revealed the degradation of IκB-α albeit to a lower extent (data not shown). In addition, PANC-1 cells (2-ME responsive) also demonstrate similar down regulation of IκB-α (Figure 4A). On the contrary, in Hs 766T cells which does not respond to 2-ME (1, 2), we do not observe any alteration in either GR (Figure 3B) or IκB-α level (Figure 4B).

5. DISCUSSION

The development of malignancies is a multi-step process that comprise of initiation, progression, invasion and finally establishment of metastasis. The multiple genetic alterations at each stage impart selective advantage over normal counterpart. In this respect, pancreatic

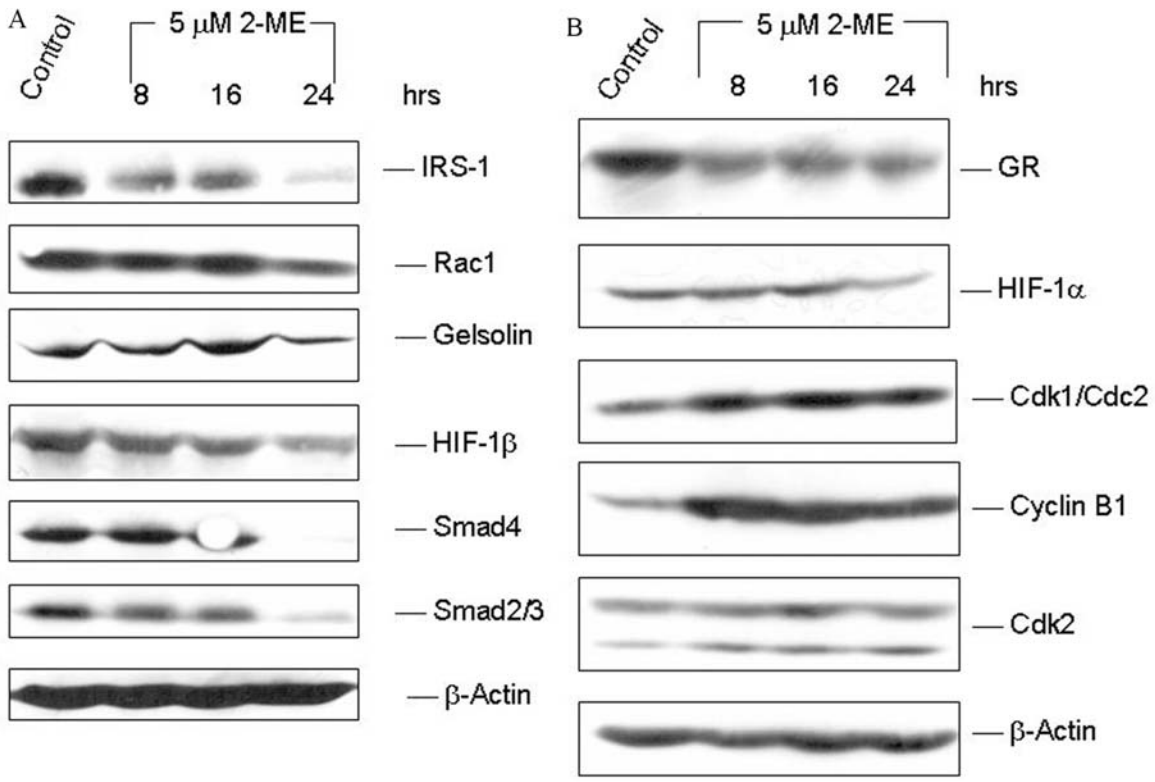

Figure 2. Conventional Western blotting used for validation of Powerblot data. Control cells and cells treated with designated amount of estrogen metabolite were processed for Immunoblotting using antibodies (BD Biosciences Laboratories) against the indicated proteins. For all the blots, equal loading in each lane was verified by Ponceau S staining. Furthermore, β-Actin level was monitored in these samples as internal control.

carcinoma is no exception. During last decade, several cancer-causing genes have been found to be associated with pancreatic carcinoma (54). Undoubtedly, the identification of these cancer related genes have substantially improved our knowledge on pancreatic cancer development. However, the treatment of this cancer has not advanced that much. This might be due to the lack of early diagnosis and effective chemotherapeutic treatments. In pursuit to achieve a greater survival rate of pancreatic cancer patients, new molecular targets for drug discovery or better diagnosis markers are necessary at this stage.

2-Methoxyestradiol (2-ME) is in clinical trials to evaluate its activity against breast cancers and multiple myelomas (9, 12). However, the studies on its effect on pancreatic carcinoma are limited to a few *in vitro* or preclinical studies (1, 2, 55, 56). Cumulative studies demonstrate that multifunctional behavior of 2-Methoxyestradiol on cell proliferation, angiogenesis and apoptosis are mediated by modulation of multiple signal transduction pathways. In the present study, using large scale proteomic analysis we have identified altered profile of several proteins in 2-ME exposed pancreatic cancer cells. The observations reported here might allow defining potential new molecular target (s) of this estrogen metabolite. For instance, as shown in Fig.2, the level of Rac1 protein is markedly attenuated after continued 2-ME exposure for 24 hrs. Rac1 is a member of Rho GTPase family proteins, which are localized at membranes and become activated upon stimulation of cell surface receptors. Activated Rho proteins bind to effector molecules to trigger specific cellular responses such as cell motility, cell-cell and cell-extracellular matrix adhesion as well as cell cycle progression. Each of these functions plays a pivotal role for the development and progression of cancer. Mutated form of Rac1 confers tumorigenicity in mice and may contribute to the acquisition of metastatic phenotype *in vivo (*57). Also dysregulated expression of Rac1 was shown to be associated with pancreatic adenocarcinoma (58). The finding that 2-ME can attenuate the expression of Rac1 (Figure 2A) has potential therapeutic benefit, because inhibitors of Rho proteins appear to be promising targets for the development of novel anticancer drugs.

To the other end, the level of gelsolin, a major substrate of caspase-3, is also diminished under 2-ME stress (Figure 2A). Gelsolin was identified as a result of its ability to sever actin filaments in a Ca^{2+}- and pH-dependent manner (59). It is believed that uncleaved full-length gelsolin is capable of inhibiting release of cytochrome c and subsequent apoptosis by interacting with mitochondrial voltage-dependent anion channels (59).

Another important element of our investigation is the down regulation of Smads by 2-ME in MIA PaCa-2 cells. Smad genes are key transducers of information

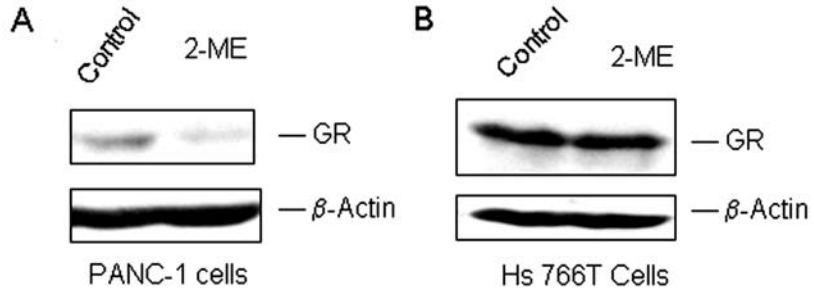

Figure 3. Proapoptotic effect of 2-ME is correlated with GR down regulation. A & B represent immunoblot analyses of designated cell extract with GR and β-Actin specific antibodies. Pancreatic cancer cells were either treated with vehicle solvent (DMSO) or 5 μM 2-ME for 24 hrs. Of note, diminished levels of GR are evident in 2-ME sensitive PANC-1 cells (Panel A) but not in 2-ME non responsive Hs766T cells (Panel B).

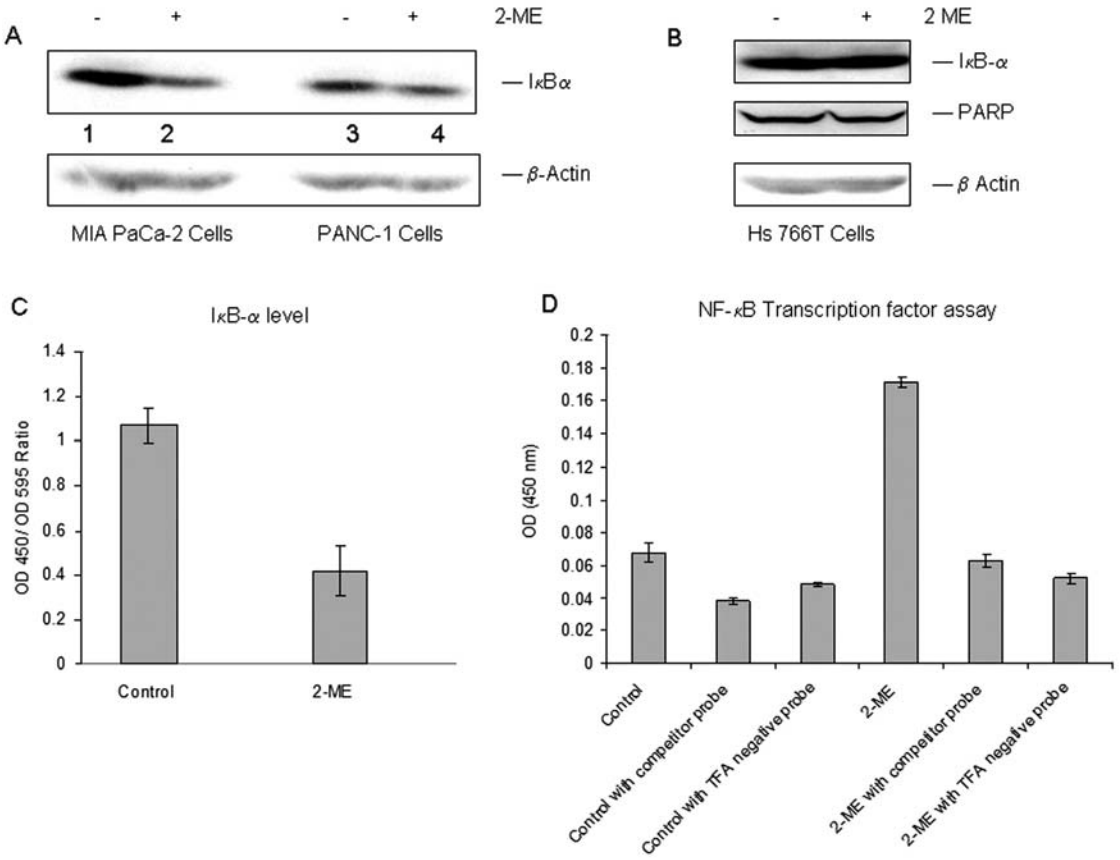

Figure 4. Enhanced NF-κB activity in 2-ME exposed MIA PaCa-2 pancreatic cancer cells. A & B, Western blot analysis of IKB-α /PARP protein in untreated and 2ME treated cell extract. IkBα antibody (Cell Signaling). C, Quantitation of total IkB-α by cell based ELISA. Cells were seeded at a density of 1.5 x10^4 cells per well. Control and 2-ME treated cells were fixed with formaldehyde. Incubation with Primary and Secondary antibodies, colorimetric detection (OD 450) as well as determination of relative cell numbers (OD 590) were carried out as described in Materials and Methods. Ratio of absorbance at OD 450 and 590 was compared between control and 2-ME exposed cells ($P = 0.0002$). D, NF-κB transcription factor assay. DNA binding activity of NF-κB was measured in nuclear extract isolated from control and treated cells as described above. Briefly, the bound NF-κB was detected with rabbit anti- NF-κB p65 primary antibody and a highly sensitive HRP-conjugated secondary antibody. The colorimetric detection in 96 well format was carried out using a spectrophotometric plate reader. The specificity of DNA binding was assured by performing the assay in the presence of excess NF-κB specific competitor oligos. Transcription factor assay (TFA) negative control probe, which does not contain NF-κB consensus sequence, was used as internal negative control. For A, B &C, cells were treated with 5 μM 2-ME for 16 hrs. 2-ME induced NF-κB activation was statistically significant ($P < 0.0001$) when compared to that of control.

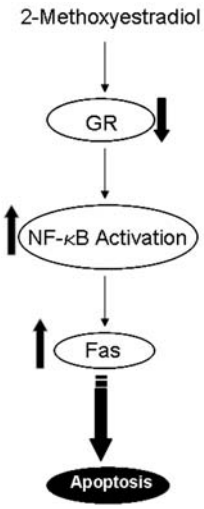

Figure 5. Schematic presentation of possible cross-talk between GR and NF-κB in 2-ME mediated apoptosis of MIA PaCa-2 cells.

transmitted by TGF-β. The inactivation of Smads by mutation can render enhancement of tumor growth in majority of pancreatic neoplasms (50) and thus diminished level of mutated Smads might favor the antiproliferative action of 2-ME in pancreatic cancer.

Most importantly, the studies depicted here suggest the down regulation of glucocorticoid receptor (GR) previously not reported in relation to proapoptotic effect of 2-ME. Of note, earlier reports described varying specificity of the transcriptional activity of GR between cell types, leading to diverse and opposite physiological effects of glucocorticoid in different tissues. In contrary to lymphocytes where glucocorticoids have been shown to promote apoptosis, human mammary epithelial cells and rat hepatoma epithelial cells are protected from apoptosis after GR activation (60,61).

One prominent candidate gene for anti-apoptotic regulation by glucocorticoid (GCs) is the transcription factor NF-κB, involved in the regulation of diverse apoptosis genes such as FasL/CD95-L, Fas/CD95, DR4 and DR5 that all carry NF-κB consensus sequences in their promoter regions. In addition, GCs can repress transcription of target genes by transcriptional interference, a mechanism likely to involve protein-protein interactions between GR and NF-κB (41-43). Here we demonstrate that the activation of NF-κB is concomitant with 2-ME mediated cytotoxicity of pancreatic cancer cells. Of note, in line with this observation, 2-ME like tubulin binding agent paclitaxel or epothilones also can induce apoptosis by activation of NF-κB in solid tumor cells (62,63). Furthermore, GCs antagonized paclitaxel-mediated NF-κB activation through induction of IκB synthesis.

In our laboratory, we previously observed that 2-ME induced up regulation of Fas as well as the adapter protein FADD, might play a pivotal role in initiating apoptotic cascade in pancreatic cancer cells MIA PaCa-2 (1). Collectively, it is reasonable to speculate that upstream events such as GR down regulation and enhanced NF-κB activity are predominant regulators of induction of Fas during apoptotic cell death caused by 2-ME. While NF-κB is constitutively activated in numerous tumors, this tumor microenvironmental molecule also plays an antimalignancy role in various tumors. Similar to other microenvironmental molecules such as TGF-β or TNFα, NF-κB also bears double edged sword functions by playing opposing antiapoptotic and proapoptotic roles depending on the cell type (28,37,38).

It is apparent that NF-κB cannot be induced or inhibited by 2-ME in metastatic pancreatic carcinoma cells Hs766T (Figure 4B). Simultaneously, 2-ME cannot modulate GR level in these cells (Fig.3B). It remains to be seen whether lack of interplay between NF-κB and GR plays significant role in 2-ME resistance in these cells. In another scenario, p53 induction contributes to 2-ME-induced apoptosis in LNCaP cells and NF-κB activation is necessary for 2-ME-mediated p53 induction and apoptosis (64). However, we excluded this possibility because both MIA PaCa-2 and PANC-1 cells bear mutant p53 (65, 66). In the case of pancreatic cancer cells apparent functional cross-talk between NF- κB and GR (41-43) might modulate 2-ME mediated cell demise (Figure 5).

To summarize, the outcome of our investigation could possibly be helpful to better design therapeutic strategies and to unleash the pro-apoptotic role of NF- κB in 2-ME- mediated pancreatic cancer cell death**.** In many cases NF-κB is necessary for cell survival in culture, and therefore has the potential to contribute to cellular transformation, tumor progression and drug resistance. Given its opposing, pro-apoptotic activity in pancreatic carcinoma cells MIA PaCa-2 or PANC-1, therapeutic strategies designed to inhibit NF-κB must be cautiously approached. In future, it will be important to firmly establish whether NF-κB and GR compete with each other within the pancreatic tumor specimens and whether differences in activation are associated with stage and histopathology grade.

6. ACKNOWLEDGMENTS

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