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2-Methoxyestradiol modulates β-catenin in prostate cancer cells: A possible mediator of 2-methoxyestradiol-induced inhibition of cell growth

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2-Methoxyestradiol (2-ME_2) is a novel anticancer agent because of its ability to potentiate apoptotic cell death and inhibit cancer cell growth and angiogenesis. The modes of action of this agent, however, have not yet been fully elucidated. In our study, we have investigated whether 2-ME2 is able to modulate β-catenin signaling in prostate cancer cells, which is one of the major players in cell–cell adhesion, proliferation, apoptosis and carcinogenesis. We found that β-catenin levels were significantly upregulated by 2-ME_2 in a dose-dependent manner in androgen dependent and independent prostate cancer total cellular extracts. We further show that β-catenin levels were significantly increased in the membrane fraction, while nuclear fractions of β-catenin were downregulated in the 2-ME_2-treated cells. Accumulation of dephospho-β-catenin (nondegraded form) parallel with Bcl-2 and Cyclin D1 downregulation was also achieved after 2-ME_2 treatment. Moreover, we demonstrate that the β-catenin production by 2-ME_2 is mediated through the MEK/ERK-2 signaling pathway. Collectively, these results suggest that the cytostatic effect of 2-ME_2 may be mediated through the prevention of the translocation of β-catenin to the nucleus parallel with an increase in cell–cell adhesion by increasing membrane β-catenin production, eventually preventing cell migration. Moreover, dephospho-β-catenin accumulation by 2ME_2 in the cytoplasm may contribute to the induction of apoptosis of these cells. Finally, studies testing the efficacy of 2-ME_2 in human prostate cancer are warranted to determine whether the inhibition of the expected loss of membranous β-catenin and the upregulation of nuclear β-catenin can prevent prostate cancer development and progression.

Key words: 2-methoxyestradiol; β-catenin; prostate cancer; MAPK signaling; apoptosis

Prostate cancer remains the most common cancer in males, with the American Cancer Society estimates of greater than 218,890 new cases diagnosed in the United States in 2007, resulting in an estimated 27,050 deaths.[1,2] Although significant advances have been made in our understanding of prostate-cancer development and progression, we greatly need new treatment and preventive strategies.

2-Methoxyestradiol (2-ME_2), a physiological metabolite of natural estrogen 17β-estradiol, is produced in mammalian cells as an important step of elimination of potentially damaging catechol estrogen from proliferating cells.[3] 2-ME_2 is a promising anticancer agent, because it reveals antiproliferative and apoptotic activities as well as antiangiogenic activity by modulating some genes and signaling the molecules associated with these events.[4–9] Moreover, it is an oral agent[8] with limited toxicity.[10] While several studies have been made on the mode of action of 2-ME_2 and provided evidence on its clinical importance, very little is uncovered about the impact of this agent on cell-adhesion molecules such as β-catenin, which are associated with carcinogenesis. Therefore, an understanding of the action of 2-ME_2 on β-catenin and its signaling pathway will help further to define its role in these new treatment and preventive approaches.

β-Catenin is a dynamic multifunctional protein involved in cell–cell adhesion, proliferation[11] and apoptosis.[12] It is a key component of the cadherin/catenin complex that mediates calcium-dependent homophilic interactions of intercellular adhesion.[13] In addition, β-catenin is a crucial downstream effector in the Wnt/Wingless signaling pathway that governs developmental processes and tumorigenesis.[14,15] In normal and nonstimulated cells, β-catenin is important in maintaining cell–cell adhesion and is localized at the cell membrane as a cadherin–catenin complex. A very low amount of β-catenin is normally located in the cytoplasmic compartment, and it is tightly controlled by multiple proteins including Smad7, which is regulated by 2-ME_2.[16] In the cytoplasm, β-catenin destabilizes and degrades through the interaction of multiple proteins such as Axin, adenomatous polyposis coli (APC) and a serine/threonine glycogen synthesis kinase (GSK)-3β.[17,18] The phosphorylation of cytoplasmic β-catenin by GSK-3β followed by a direct interaction with APC is an essential event for destabilization and destruction of β-catenin through the interaction of the β-transducin repeat-containing protein (β-TrCP).[19] The sequential events are perturbed when Wnt, *Ras* and phosphatidylinositol 3-kinase (PI3K) signaling pathways are activated. These events subsequently stabilized cytoplasmic β-catenin, which eventually translocates to the nucleus by interacting with its receptors, the TCF/LEF (T-cell factor/lymphocyte-enhancer factor) family of transcription factors.[20] This leads to the transactivation of target genes such as c-myc[21] and cyclin D1,[22] leading to increased cell proliferation. Alternatively, overexpression and accumulation of freeform of β-catenin in the cytoplasm is involved in cell-cycle arrest and enhances the apoptosis through p53-dependent[23] or -independent pathways.[12] Together, these studies indicate biphasic (*i.e.*, positive and negative) roles of this molecule on cell fate depending on the microphysiological conditions and suggest that targeting this molecule by an agent could be a novel approach to treat prostate cancers.

In this report, we show that 2-ME_2 concurrently increases β-catenin accumulation in the membrane and blocks its translocation to the nucleus parallel with the inhibition of the expression of its targeted genes such as Cyclin D1 and Bcl-2. Moreover, this modulation of β-catenin is mediated through MEK-ERK-2 signaling pathway. These studies, therefore, suggest that regulation of β-catenin signaling by 2-ME_2 in prostate cancer cells may lead to enhanced cell–cell contacts as well as apoptotic death.

Material and methods

Reagents and antibodies

Dulbecco's modified Eagle's medium (DMEM), penicillin–streptomycin, trypsin EDTA solution, anti–actin and 2-methoxyestradiol (2-ME_2) were purchased from Sigma (St. Louis, MO). Twenty micromolar stock solution of 2-ME_2 was prepared in absolute ethyl alcohol and stored at −20°C. β-Catenin antibody was purchased from BD Biosciences (San Jose, CA). TCF-3/4

Peter J. Van Veldhuizen and Gibanananda Ray contributed equally to this work.

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antibody was purchased from Upstate (Charlottesville, VA), Cyclin D1 and Bax antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Dephospho β-catenin monoclonal antibody was purchased from Alexis Biochemicals (San Diego, CA). Bcl-2 monoclonal antibody was purchased from Oncogene Research Product (San Diego, CA). MEK inhibitor (U0126) and JNK inhibitor II (SP600125) were purchased from Promega (Madison, WI) and CalBiochem (San Diego, CA), respectively. Secondary antibodies such as goat antirabbit IgG-horse radish peroxidase (HRP) and goat antimouse IgG-HRP were purchased from Santa Cruz Biotechnology. Coomassie protein assay reagent was obtained from Pierce Biotechnology (Rockford, IL). β-catenin recombinant protein was purchased from Upstate. All other chemicals were purchased either from Sigma or Fisher Scientific (Houston, TX).

Cell culture conditions and treatments

The DU145, PC-3 and LNCaP prostate cancer cell lines were obtained from American-type culture collection (ATCC, Manassas, VA). The cell lines were grown in DMEM supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 units/ml penicillin and 100 units streptomycin at 37°C, 5.0% CO_2/95% air atmosphere. The cells were cultured as monolayers in 75-cm^2 flasks. Approximately 60% confluent cells were treated with 1, 5 and 10 μM 2-ME_2 for 48 hr. Cells treated with ethanol (less than 1 ml) were considered as vehicle-treated controls.

Isolation of subcellular fractions of protein from DU145 prostate cancer cells

Membrane protein was isolated as per the manufacturer's instructions by using Mem-PER® eukaryotic membrane protein extraction reagent kit, and both nuclear and cytoplasmic proteins were extracted by using NE-PER™ nuclear and cytoplasmic extraction reagent from PIERCE (Pierce Biotechnology).

Western blot analysis

The Western blot analysis was performed as described by Banerjee *et al.*[4] Briefly, 2-ME_2-treated and untreated control cells were washed with ice-cold 1× PBS (pH 7.4), followed by incubation in cell-lysis buffer for 30 min at 4°C. Protein concentrations were measured by the Coomassie blue detection method. Twenty-five microgram total protein was separated by SDS-PAGE. The fractionated proteins were transferred to nitrocellulose membrane and subsequently probed with primary antibodies for a specific period of time as per the manufacturer's instructions. Blots were washed in TBS-T and incubated with secondary antibodies conjugated with HRP for 30 min at room temperature. Immuno reactions (antigen–antibody) were detected by using enhanced chemiluminescence (Pierce), and the intensity of the band was measured by densitometric analysis using one-dimensional image software (Version 3.6).

Results

2-ME_2-induced upregulation of β-catenin in androgen-dependent and androgen-independent prostate cancer cells

We investigated β-catenin protein status in the androgen-dependent cell line, LNCaP, and the androgen-independent cell lines, DU145 and PC-3, using immuno-Western blot analysis. As shown in Figure 1, β-catenin protein levels were found to be significantly increased in the total cell extracts of LNCaP, DU145 and PC-3 prostate cancer cells after 5 and 10 μM 2-ME_2 treatments for 48 hr as compared with the vehicle-treated control. There was a significant induction of β-catenin protein level in 1 μM 2-ME_2 exposed PC3 cells. However, this effect was undetected in LNCaP and DU145 cells (Fig. 1). This finding suggests that irrespective of androgen sensitivity, 2-ME2, at higher doses, upregulates β-catenin expression in all 3 prostate cancer cells.

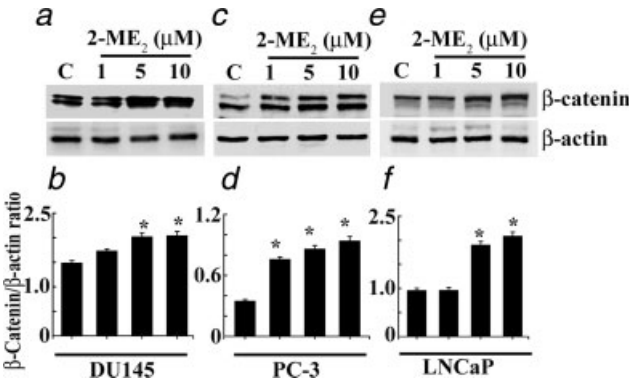

FIGURE 1 – 2-Methoxyestradiol (2-ME_2)-induced β-catenin protein expression in DU145, PC-3 and LNCaP prostate cancer cells. Total protein was isolated and immuno-Western blot analyses were performed using mouse monoclonal antibodies. (*a*, *c* and *e*) Single representative blots showing β-catenin and β-actin (internal control) levels in vehicle-treated control; (C) 1, 5 and 10 μM 2-ME_2-treated DU145, PC-3 and LNCaP prostate cancer cells, respectively; (*b*, *d* and *f*) The bar diagrams show mean ± SD ratio of β-catenin and β-actin of 3 independent experiments. *$p < 0.005$, *versus* untreated control (Student's *t*-test).

2-ME_2 abrogates the nuclear translocation of β-catenin

β-Catenin is a dynamic protein that, under pathological conditions, enters the nucleus and binds with TCF/LEF-1 proteins, resulting in the transactivation of target genes associated with the cellular proliferation.[22] Several studies, including our recently published data, indicate that 2-ME_2 prevents prostate cancer cell proliferation.[15,24,25] Building on these results, we sought to explore whether 2-ME_2 is able to prevent the translocation of β-catenin from the cytoplasm to the nucleus. To do so, DU145 cells were exposed to 5 μM 2ME_2 for 48 hr. Proteins were extracted from different cellular compartments including nucleus, cytoplasm and membrane, and expression profiles of β-catenin were evaluated in different fractions by immuno-Western blot analysis using an antibody to β-catenin. As shown in Figure 2, the β-catenin level in nuclear fraction was significantly decreased in 2-ME_2-treated cells as compared with the vehicle-treated control. We found that 5 μM 2ME_2 markedly reduced the translocation of β-catenin in DU145 cells after 48 hr of treatment, while it elevated significantly the membrane β-catenin fractions in the same samples. However, no significant change was observed in cytoplasmic β-catenin expression (Fig. 2). Because the effects of higher doses of 2-ME2 on β-catenin in DU145, PC3 and LNCaP were identical, our study and subsequent studies were carried out with DU145 cells.

Effect of 2-ME_2 on phosphorylation of β-catenin and TCF expression in prostate cancer cells

The accumulation or degradation of β-catenin in the cytoplasm is strictly dependent upon the phosphorylation at the amino-terminal region of this protein.[26–28] The nonphosphorylated β-catenin either activates targeted genes through the association with TCF/LEF-1 transcription factors[26] or induces apoptosis through complex TCF/LEF-1-independent pathways.[12,29–31] In this set of experiments, first we determined whether 2-ME_2 is able to modulate the phosphorylation status of the β-catenin protein in DU145 prostate cancer cells. To test this, the phosphorylation level was determined in 2-ME_2-treated and untreated cells by immuno-Western blot analysis using the dephospho β-catenin antibody (aa 27–37). We found that the dephospho β-catenin protein levels were markedly increased in 1, 5 and 10 μM 2-ME_2-treated total cell extracts for 48 hr as compared with the vehicle-treated controls (Fig. 3). Next, we determined the status of the β-catenin receptor TCF-3/4 in DU145 cells after the treatment with 2-ME_2. The rationale of the selection of the TCF-3/4 transcription factor

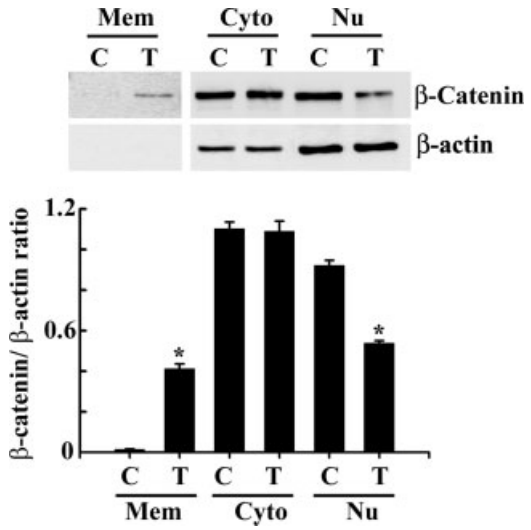

FIGURE 2 – 2-Methoxyestradiol (2-ME$_2$)-induced modulation of β-catenin in subcellular protein fractions in DU145 prostate cancer cells. Approximately 60% confluent cells were treated with 2-ME$_2$ for 48 hr. Membrane, cytoplasmic and nuclear protein fractions were isolated, and immuno-Western blot analyses were performed using specific antibodies. The representative blots show β-catenin and β-actin levels in vehicle-treated control (C) and 5 μM 2-ME$_2$ treated (T) in membrane (Mem), cytoplasmic (Cyto) and nuclear (Nu) fractions (Top), and the bar diagram shows mean ± SD ratio of β-catenin and β-actin of 3 independent experiments (bottom). *, $p < 0.005$, *versus* control (Student's *t*-test).

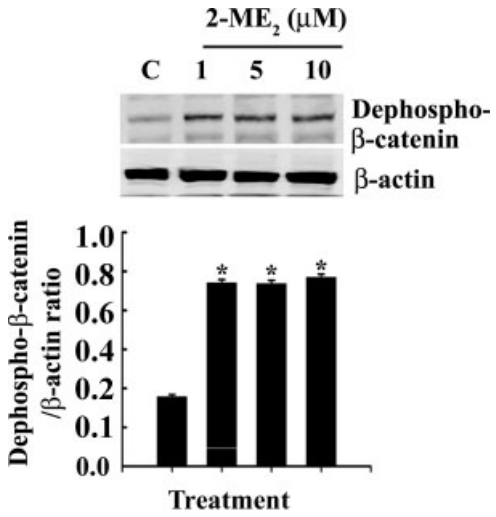

FIGURE 3 – 2-ME$_2$-induced modulation of dephospho-β-catenin in DU145 cells. Total protein was isolated and immuno-Western blot analyses were performed using antidephospho β-catenin (aa 27–37) monoclonal antibody. Blots were then stripped and β-actin was done for the internal controls. The representative blots show dephospho β-catenin and β-actin levels in control (C), 1, 5 and 10 μM 2-ME$_2$-treated prostate cancer cells (top), and the bar diagram shows mean ± SD ratio of dephospho β-catenin and β-actin of 3 independent experiments. *$p < 0.01$ *versus* untreated control (Student's *t*-test).

for our study was that these factors (TCF-3 and TCF-4) are members of the TCF/LEF family of transcription factors and, along with β catenin, they are able to transactivate the downstream genes of Wnt signaling in different epithelial cell types.[32–35] We found that TCF-3/4 levels were not modulated by 2-ME$_2$ in these cells (data not shown). Together, these studies suggest β-catenin regulation by 2-ME$_2$ may be associated with apoptosis. To assess this hypothesis, we determined the expression profile of antiapoptotic protein Bcl-2 and proapoptotic protein Bax in these cellular extracts. The immuno-Western blot analyses revealed that 2-ME$_2$ dose dependently inhibits Bcl-2 production (Fig. 4) in these cells without altering the production of Bax (data not shown).

2-ME$_2$ inhibits β-catenin-induced cyclin D1 production in DU145 prostate cancer cells

Because 2-ME$_2$ decreased nuclear β-catenin in prostate cancer cells, it might act as an inhibitor of β-catenin-TCF-3/4-dependent regulatory genes. To address this possibility, we investigated whether 2-ME$_2$ could impair the β-catenin-dependent expression of Cyclin D1, the cell-cycle regulatory protein. DU145 cells at 60% confluence were exposed to recombinant β-catenin protein alone or β-catenin protein plus 2-ME$_2$ (5 μM) for 48 hr, and Cyclin D1 levels were determined by immuno-Western blot analysis using Cyclin D1 specific antibodies. Cyclin D1 levels were significantly higher in β-catenin exposed cells as compared to unexposed cells, and this induction can be abolished by 2-ME$_2$ treatment (Fig. 5). These studies suggest that the 2-ME$_2$-induced inhibition of the translocation of β-catenin and its expression limits the transactivation action of β-catenin.

β-Catenin in DU145 prostate cancer cell is regulated via *the MEK-ERK 2 signaling pathway*

Our previous data indicate that 2-ME$_2$ induced phosphorylation of JNK and ERK2 is correlated with apoptosis in DU145 cells.[25] Next, we determined whether these signal transduction pathways are involved in the differential regulation of β-catenin by 2-ME$_2$.

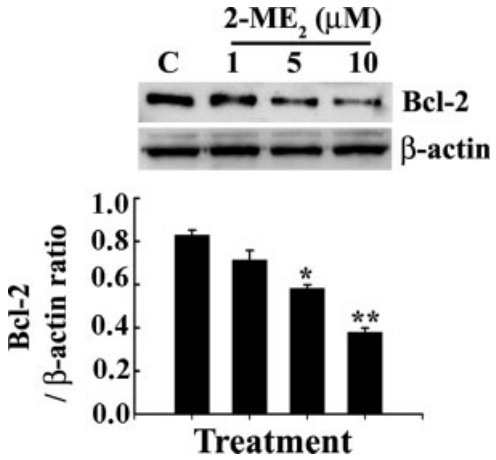

FIGURE 4 – Effect of 2-ME$_2$ on Bcl-2 in DU145 cells. Semiconfluent cells were treated with indicated concentrations of 2-ME$_2$ or vehicle (ethanol) for 48 hr. Total protein was isolated and immuno-Western blot analyses were performed using anti-Bcl-2 antibody. Western blotting with an anti-β-actin antibody on a stripped blot revealed that an equal amount of protein was loaded in each lane. The bar diagram shows mean ± SD ratio of Bcl-2 and β-actin of 3 independent experiments. *$p < 0.05$, **$p < 0.005$, *versus* vehicle-treated control (C) (Student's *t*-test).

To test this, ~60% confluent DU145 cells were exposed to either 5 μM 2-ME$_2$ alone or its combination with MEK inhibitor U0126 or JNK inhibitor SP600125 for 48 hr. The stimulation of β-catenin by 5 μM 2-ME$_2$ in DU145 cells can be inhibited to the basal level or less by the MEK inhibitor (Fig. 6). This effect was not significant when cells were treated with SP600125 (data not shown).

Discussion

Despite the complex and bifunctional behavior of 2-ME$_2$, it is considered a promising anticancer drug.[5] Consistent with other works, our prior studies have shown that 2-ME$_2$ inhibits cell

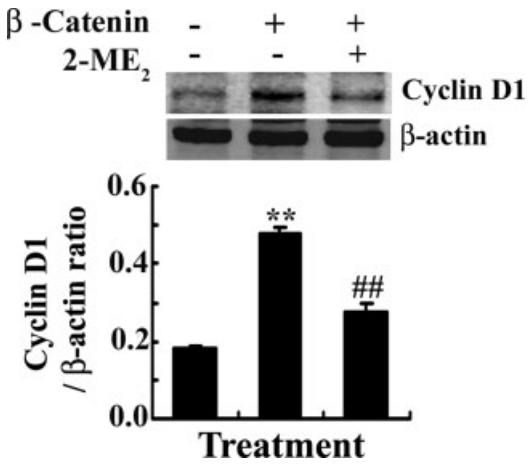

FIGURE 5 – Effect of 2-ME_2 on β-catenin-induced CyclinD1 expression in DU145 cells. Semiconfluent cells were treated with recombinant β-catenin protein (100 ng/ml) or β-catenin protein plus 2-ME_2 (5 μM) or vehicle-treated (ethanol control, which is denoted as negative sign) for 48 hr. Total protein was isolated and Cyclin D1 levels were determined by immuno-Western blot analysis using Cyclin D1 specific antibodies. Western blotting with an anti-β-actin antibody on a stripped blot revealed that an equal amount of protein was loaded in each lane. The bar diagram shows mean ± SD ratio of Cyclin D1 and β-actin of 3 independent experiments. $^{**}p < 0.001$, *versus* ethanol-treated control $^{\#\#}p < 0.001$, *versus* β-catenin-treated (Student's *t*-test).

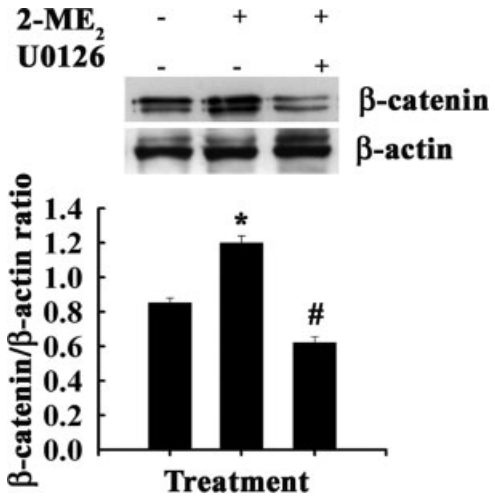

FIGURE 6 – MEK/ERK inhibitor (U0126) modulates 2-methoxyestradiol (2-ME_2)-induced β-catenin expression in DU145 prostate cancer cells. Approximately 60% confluent cells were treated for 48 hr with 5 μM 2-ME_2 alone or after 30 min of U0126 exposure. Total protein was isolated and separated by SDS-PAGE followed by immuno-Western bolt analysis for the detection of β-catenin or β-actin expression. The bar represents the mean ± SD level of β-catenin to β-actin ratio of 3 separate experiments. *, $p < 0.05$, *versus* untreated control, #, $p < 0.005$, *versus* 5 μM 2ME_2 (Student's *t*-test).

growth and potentiates apoptotic cell death in various cancer cells *in vitro* and *in vivo*.[4,25,36] Although the mode of action of this estrogen metabolite has not yet been fully elucidated, multiple studies have demonstrated that 2-ME_2 modulates several crucial genes linked with the cell-cycle regulation.[4,5,16,25,37–45] In our study, for the first time, we postulate the possible involvement of β-catenin and its receptor, TCF-3/4 transcription factor, in the significant effect of 2-ME_2 on prostate cancer cells.

β-Catenin plays critical roles in cell–cell adhesion, cell proliferation and apoptosis. In conjugation with the intracellular binding partner E-cadherin it mediates cell–cell adhesion.[46–48] However, when β-catenin frees from its intracellular binding partner it translocates to the nucleus through the binding with TCF/LEF and is involved in the transcriptional regulation of specific genes associated with differentiation, proliferation and malignant transformation.[46,48–50] We found that 2-ME_2 dose dependently modulates β-catenin without affecting its receptor expression in prostate cancer cells. 2-ME_2 induces total and membrane β-catenin, while inhibiting the migration of this protein to the nucleus. Thus, our study suggests that 2-ME_2-induced higher expression of membrane β-catenin may result in an increased interaction of β-catenin with E-cadherin, which may eventually lead to an overall induction of intercellular integrity. Moreover, the reduction in nuclear β-catenin translocation may minimize the formation of the β-catenin-TCF-3/4 complex, which is needed to transactivate genes for cellular proliferation. Consistent with this hypothesis, in the present study we found that beta-catenin-induced Cyclin D1 expression was blocked by 2-ME2 treatment (Fig. 5).

β-Catenin, in the absence of a stimulant such as *Wnt* or Wingless, is persistently ubiquitinated and degraded by the proteosomes. This degradation of β-catenin is dependent on the phosphorylation at the aminoterminal region of this protein.[26–28] Extracellular signaling such as *Wnt*/Wingless inhibits the phosphorylation event and induces the accumulation of cytosolic β-catenin, which eventually activates targeted genes through the association of TCF/LEF.[26] Besides the earlier roles, a contrast function has been proposed by multiple studies for β-catenin and TCF/LEF interactions in apoptosis.[12,29,30] They found that overexpression of β-catenin induces apoptosis in multiple systems under certain circumstances including LEF-independent pathways.[31] Evidently these studies indicated that the blockade of β-catenin degradation, cytoplasmic accumulation and its subsequent aberrant nuclear translocation leads to G2/M arrest,[51] which eventually leads to apoptosis.[51] Because 2-ME_2 induces apoptosis in prostate cancer cells,[25] we here investigated the particular events in 2-ME_2-exposed prostate cancer cells. We demonstrate that 2-ME_2 blocks phosphorylation of β-catenin, and that this change correlates with a significant reduction of Bcl-2 production in prostate cancer cells (Fig. 4). Therefore, we suggest that the activation of the apoptotic switch by 2-ME_2 could be mediated by increasing the freepool of β-catenin by preventing the degradation of this protein. However, further studies are warranted.

Our recent data indicated that 2-ME_2 induces cell cycle arrests in DU145 prostate cancer cells through both JNK and ERK 2 signaling pathways.[25] These observations led us to determine whether β-catenin is regulated through these signaling pathways. The upregulation of β-catenin in 5 μM 2-ME_2-treated cells was inhibited when cells were treated with U0126, an inhibitor of MEK, in combination with 5 μM 2-ME_2 as compared to the untreated control (Fig. 6). Thus, we suggest that β-catenin is regulated through MEK/ERK 2 signaling pathway and indicate that β-catenin is one of the downstream effectors of MEK/ERK 2.

In summary, these findings suggest that 2-ME2 may enhance apoptotic cell death or inhibit proliferation by preventing the translocation of β-catenin to the nucleus parallel with an increase in cell–cell adhesion by increasing the expression of membrane β-catenin. Moreover, accumulation of dephospho-β-catenin (nondegraded form) by 2-ME_2 treatment may contribute to the induction of apoptosis. Finally, studies testing the efficacy of 2-ME_2 in human prostate cancer are warranted to determine whether the prevention of the expected loss of membranous β-catenin and the upregulation of nuclear β-catenin can prevent prostate cancer development and progression.

Acknowledgements

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