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Ionomycin downregulates β-catenin/Tcf signaling in colon cancer cell line

Chi Hoon Park, Eun Ryeong Hahm, Ju Hyung Lee, Kyung Chae Jung, Ho Sung Rhee and Chul Hak Yang*

Division of Chemistry and Molecular Engineering, Seoul National University, Seoul 151-742, Korea

*To whom correspondence should be addressed. Tel: +82 2 878 8545; Fax: +82 2 889 1568;
Email: chulyang@plaza.snu.ac.kr

Functional activation of β-catenin/Tcf signaling plays an important role in the early events in colorectal carcinogenesis. We examined the effect of ionomycin against β-catenin/Tcf signaling in colon cancer cells. Reporter gene assay showed that ionomycin inhibited β-catenin/Tcf signaling efficiently. In addition, the inhibition of β-catenin/Tcf signaling by ionomycin in HEK293 cells transiently transfected with a constitutively mutant β-catenin gene, whose product is not phosphorylated by GSK3β, indicates that its inhibitory mechanism is related to β-catenin itself or downstream components. To investigate the precise inhibitory mechanism, we performed immunoprecipitation analysis, western blot and electrophoretic mobility shift assay. As a result, our data reveal that the association of β-catenin and Tcf-4 is disrupted and the amount of β-catenin product in the nucleus is decreased by ionomycin in a concentration-dependent manner. Moreover, ionomycin strongly suppressed the binding of the Tcf complexes to its specific DNA-binding sites. The significance of the current work is that ionomycin is a negative regulator of β-catenin/Tcf signaling in colon cancer cells and its inhibitory mechanism is related to the decreased nuclear β-catenin products and to the suppressed binding of Tcf complexes to consensus DNA.

Introduction

β-Catenin, which is a 92–97 kDa protein, has been shown to have a dual role as a major structural component of cell–cell adherens junctions and as a transcription activator in the nucleus. It associates with the cytoplasmic domains of E-cadherin and links them, through α-catenin, to the actin cytoskeleton (1,2). This cytoskeletal link can be damaged by tyrosine phosphorylation of E-cadherin-associated catenins resulting in reduced cell–cell adhesion (3,4). In addition, β-catenin is involved in the Wnt signaling pathway that regulates developmental processes in a variety of organisms, and in tumorigenesis by transactivating the lymphoid enhancer factor/T-cell factor (Lef/Tcf) transcription factor (5–7). Some of the genes activated by β-catenin/Tcf signaling are *c-jun*, *c-myc*, *fibronectin*, *cyclin D1* and *fra-1* (8–10). In normal cells, most β-catenin protein is present at cell–cell junctions with very little in cytoplasm or the nucleus because of the ubiquitin-mediated proteolytic degradation. When β-catenin exists freely in cytoplasm, it is phosphorylated by axin-adenomatous polyposis coli (APC)–glycogen synthase kinase 3β (GSK3β) complex and recognized by β-TrCP, an F-box component of the E3 ubiquitin ligase complex that promotes ubiquitination of β-catenin and increases its susceptibility to degradation by the ubiquitin-proteasome system (11). When the failure of this degradation in cells occurs, β-catenin proteins accumulate in cytoplasm and some of them translocate into the nucleus leading to the activation of Tcf/Lef transcription factor. Activated β-catenin/Tcf signaling due to the accumulation of β-catenin in the nucleus has been implicated in human carcinogenesis. This failure of degradation may result from the mutation of either the β-catenin gene itself or the tumor suppressor gene APC. The APC gene or the GSK3β phosphorylation site within exon 3 of the β-catenin gene is mutated in many cancer cells including colorectal cancer, melanoma, hepatocellular carcinoma and gastric carcinoma, resulting in the activated transcriptional activity of β-catenin/Tcf signaling (12–14). Especially, in sporadic colorectal cancers, mutations in APC have been identified in up to 80%. In addition, half of a large group of sporadic colorectal cancers and colorectal cancer cell (CRC) lines lacking APC mutations were shown to possess somatic mutations in the β-catenin (15,16). This means that the dysregulation of β-catenin plays a crucial role in colon cancer cells. Therefore, if β-catenin's transcriptional activity can be markedly downregulated, tumor growth will be suppressed.

Ionomycin is a representative calcium ionophore. Recently, ionomycin has been reported to induce apoptosis in various cancer cells by increasing the free Ca^{2+} concentration and consequently activating endonucleases to degrade nucleosomal DNA (17–20). In addition, Miyake *et al*. (21) have reported the synergistic effect between ionomycin and a chemotherapeutic agent to further suppress tumor growth. To elucidate why intake of ionomycin is disadvantageous to cancer cells, we investigated the effect of ionomycin on β-catenin/Tcf signaling in colon cancer cells. In this paper, we investigate whether ionomycin inhibits β-catenin/Tcf signaling in colon cancer cells and the mechanism by which ionomycin acts on β-catenin/Tcf transcriptional activity.

Abbreviations: APC, axin-adenomatous polyposis coli; CRC, colorectal cancer cell; EMSA, electrophoretic mobility shift assay; GSK3β, glycogen synthase kinase 3β; TRE, Tcf response elements.

Materials and methods

Cell lines and reagents

HCT116 and HEK293 cell lines were derived from KCLB (Korean Cell Line Bank, Korea). Ionomycin was purchased from Sigma (Missouri, USA). Stock solutions (5 mM) were made in dimethyl sulphoxide (DMSO). TOPflash was provided by Hans Clevers via Bart O.Williams and mutant β-catenin gene (pcDNA S33Y) was provided by Eric R.Fearon.

Isolation of cellular and nuclear extracts

Cells were trypsinized and whole cell proteins were obtained by lysing the cells on ice for 20 min in 700 μl of lysis buffer (0.05 M Tris–HCl, pH 7.4,

0.15 M NaCl, 1% NP-40, 0.5 M polymethyl sulphonyl fluoride, 50 μg/ml aprotinin, 10 μg/ml leupeptin, 50 μg/ml pepstatin, 0.4 mM sodium orthovanadate, 10 mM sodium fluoride and 10 mM sodium pyrophosphate). The lysates were then sonicated for 20 s and spun at 15 000 *g* for 10 min, and the supernatant was saved. Protein determinations were made using the method of Bradford. The nuclear pellets were prepared by resuspending cells in 800 μl of lysis buffer (10 mM HEPES, pH 7.8, 10 mM KCl, 2 mM $MgCl_2$ and 0.1 mM EDTA), placing them on ice for 15 min and then vigorously mixing after the addition of 50 μl of 10% NP-40. After a 30 s centrifugation (16 000 *g* at 4°C), the pelleted nuclei were resuspended in 120 μl of extraction buffer (50 mM HEPES, pH 7.8, 50 mM KCl, 300 mM NaCl, 0.1 mM EDTA and 10% glycerol) and incubated on ice for 30 min. Nuclear extracts were stored at −70°C.

Immunoprecipitation

Nuclear lysates were prepared and 1 ml lysates were incubated with 5 μl primary antibody for 90 min at 4°C. To this, 30 μl protein A/G plus (Santa Cruz, USA) was added and the complex was incubated at 4°C overnight. Washed the pellet three times with high salt buffer (1 M Tris–HCl, pH 7.4, 0.50 M NaCl and 1% NP-40) and three times with lysis buffer. The immunoprecipitated complexes were released with 2× sample buffer for western analysis.

Western analysis

Western analysis for the presence of β-catenin and cyclin D1 was performed on the whole extract, or nuclear proteins from HCT116 and HEK293 cells. Whole cell and nuclear extracts were isolated as described above. Protein (20–100 μg) was mixed 1:1 with 2× sample buffer (20% glycerol, 4% SDS, 10% 2-ME, 0.05% bromophenol blue and 1.25 M Tris–HCl, pH 6.8) loaded onto a 12% SDS–PAGE gel, and run at 120 V for 2 h. Cell proteins were transferred to nitrocellulose (ECL; Amersham, Arlington Heights, IL) for 3 h at 250 mA. Equal loading of the protein groups on the blots was evaluated using Bradford assay, control band and PonceauS staining. The nitrocellulose was then blocked with 5% milk in TBST overnight, washed four times, and then incubated with the primary Ab (Santa Cruz Biotechnology, Santa Cruz, CA) for 2 h at room temperature. The blots were washed four times with TBST and incubated for 1 h with HRP-conjugated anti-IgG AB (Santa Cruz Biotechnology). Immunoreactive bands were developed using a chemiluminescent substrate (WEST-ZOL plus, iNtRON BioTechnology, Korea). An autoradiograph was obtained with exposure times of 2 min to 12 h.

Electrophoretic mobility shift assay (EMSA)

Nuclear extract, prepared as described above, was incubated for 30 min at room temperature with inhibitor and ^{32}P-labeled double-stranded oligomer probe (5′-CCCTTTGATCTTACC-3′). It was analyzed by non-denaturing 4% PAGE in 0.5× TBE buffer at 100 V for 40 min. Gels were visualized by autoradiography.

Transfection and luciferase assay

Transient transfection was performed using lipofectamine and plus reagent (Invitrogen, USA). Briefly, 6.5×10^5 cells (HEK293) or 1.5×10^6 cells (HCT116) were respectively seeded in the medium onto 60 mm dish. After 24 h, cells were transfected with 1.5 μg of the luciferase reporter constructs (TOPflash) and 1.5 μg of β-galactosidase gene for normalization. Especially, HEK293 cells were cotransfected with one another gene, 1.5 μg pcDNA S33Y gene. After 3 h of post-transfection, inhibitor was added with medium containing fetal bovine serum. Cells were incubated for 24 h, lysed in Reporter lysis buffer (Promega, USA) and collected for assays of luciferase and galactosidase activity, respectively.

Results

Ionomycin downregulates β-catenin/Tcf signaling in colon cancer cell line

To investigate the effect of ionomycin on β-catenin/Tcf signaling, we used HCT116 human colon cancer cells (Figure 1). This cell line contains a constitutively active transcriptional activity of β-catenin/Tcf, for HCT116 have an oncogenic mutation (deletion of Ser-45) of β-catenin gene (12,22). We used reporter gene constructs containing a series of either WT (TOPflash) or mutant (FOPflash) Tcf-binding sites, respectively, upstream of the luciferase gene (23). Subconfluent cells were cotransfected with TOPflash or FOPflash and pCMV-β-galactosidase to normalize for transfection efficiency and were treated with ionomycin for 24 h. It has been shown that Tcf-4 responsive reporter activity was highly active in this cell line. Figure 1 shows that ionomycin suppresses the Tcf transcriptional activity in a concentration-dependent manner. Within 24 h of treatment in HCT116, 3.0, 6.0 and 8.0 μM concentration reduced the Tcf transcriptional activity by 36 ± 5%, 50 ± 10% and 68 ± 4%, respectively compared with control. The FOPflash activity, a mutant for β-catenin/Tcf binding, remained unchanged after ionomycin treatment confirming that the functional binding of β-catenin/Tcf may be important for TOPflash. The results presented in Figure 1 show that ionomycin is a good inhibitor of β-catenin/Tcf signaling in colon cancer cells.

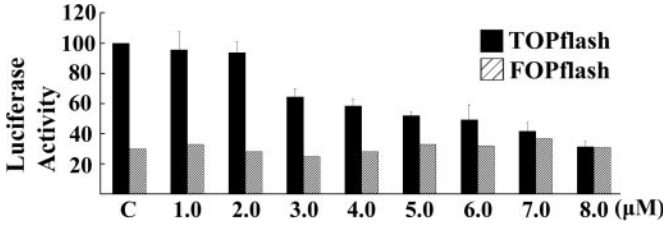

Fig. 1. Ionomycin inhibits the transcriptional activity of β-catenin/Tcf in HCT116 cells. HCT116 cells were co-transfected with reporter genes harboring Tcf-4-binding sites (TOPflash) or a mutant Tcf-binding site (FOPflash), respectively, and β-galactosidase gene. Three hours post-transfection, increasing amounts of ionomycin as indicated were added to the cells. Luciferase activity was determined 24 h post-transfection, normalized against values for the corresponding β-galactosidase activity. An equivalent volume of DMSO substituted for ionomycin was used as a vehicle control. Values represent mean ± SEM of four independent experiments.

Ionomycin acts on β-catenin or its downstream elements

To uncover the inhibitory mechanism, we investigated whether ionomycin confer their inhibitory effect upstream or downstream of β-catenin/Tcf signaling. For this purpose, we used HEK293 cells having little β-catenin/Tcf transcriptional activity. HEK293 cells were cotransfected with a constitutively active mutant of β-catenin (pcDNA S33Y), whose products are not phosphorylated by GSK3β–APC–Axin complex, together with the reporter constructs. Figure 2A shows 100-fold increase in luciferase activity when mutant β-catenin (pcDNA S33Y) was transfected into HEK293 cells compared with transfection with pcDNA (mock): 4.0 μM ionomycin inhibited the increased Tcf activity by S33Y gene transfection by ∼48 ± 2% compared with control. This strongly suggests that ionomycin exerts its inhibitory effect on β-catenin/Tcf signaling either by acting on β-catenin itself or on downstream components rather than upstream regulators. In addition, Figure 2B shows that no degradation of cellular β-catenin occurred due to ionomycin. This strongly suggests that ionomycin does not activate the degradation pathway of cytoplasmic β-catenin. The observations in Figure 2 indicate that ionomycin inhibits β-catenin/Tcf signaling either by acting on β-catenin itself or on downstream components.

Ionomycin interferes with the formation of β-catenin–Tcf complex in the nucleus

The binding of β-catenin to Tcf is inevitably required for the activation of Tcf signaling (24,25), so we examined whether ionomycin disrupts this association of β-catenin with Tcf (Figure 3). Of the several members of Tcf family, Tcf-4 is the most abundant family member in colon cells in which the Wnt pathway has been well studied (26), and so we focused on Tcf-4. HCT116 cells were treated with ionomycin at the

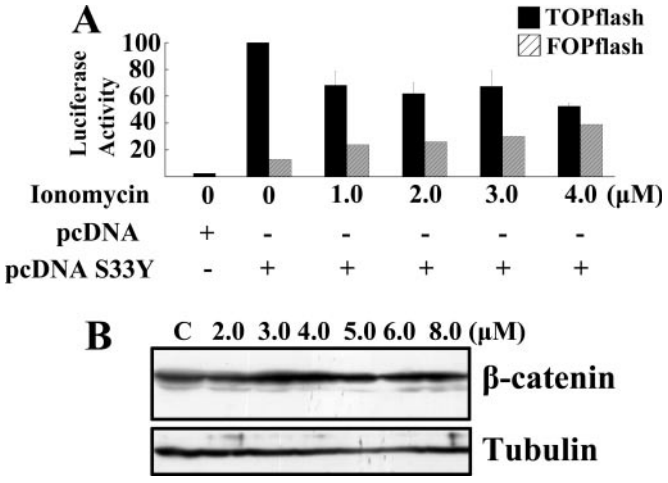

Fig. 2. The inhibition of β-catenin/Tcf signaling is not related to β-catenin degrading machinery, Axin–APC–GSK complex. (**A**) HEK293 cells were co-transfected with TOPflash (or FOPflash), β-galactosidase gene and pcDNA3.1 (vehicle) or pcDNA S33Y (mutant β-catenin gene). Three hours post-transfection, increasing amounts of ionomycin at the indicated concentration were added to the cells. Luciferase activity was determined 24 h post-transfection, normalized against values for the corresponding β-galactosidase activity. An equivalent volume of DMSO substituted for ionomycin was used as a vehicle control. Values represent mean ± SEM of four independent experiments. (**B**) HCT116 cells were incubated with ionomycin for 24 h and the cellular extractions were isolated for western blot with β-catenin antibody. To demonstrate equivalent loading of the lines, anti-tubulin was used.

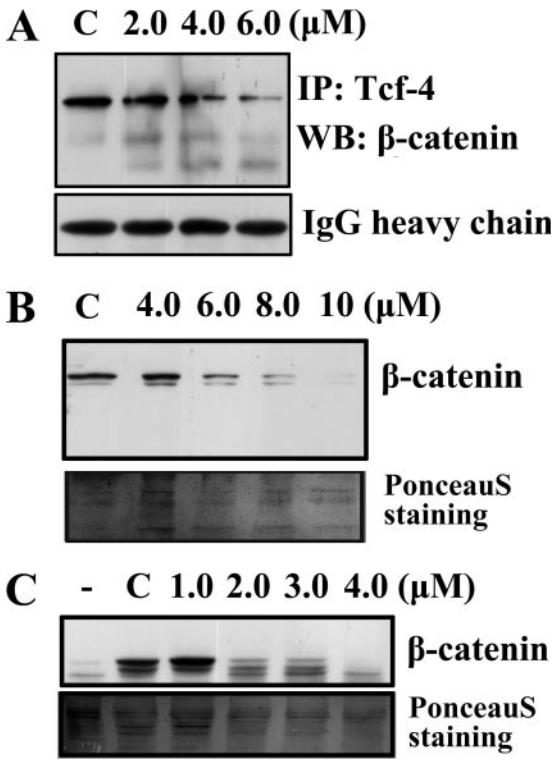

Fig. 3. The decreased nuclear β-catenin proteins by ionomycin leads to the suppressed association of β-catenin with Tcf-4. (**A**) Ionomycin was treated in HCT116 cells for 24 h. Nuclear extracts were prepared and immunoprecipitation was performed with Tcf-4 antibody followed by immunoblotting with β-catenin monoclonal antibody. IgG heavy chains were used for control band. (**B** and **C**) HCT116 (B) cells were treated with ionomycin for 24 h, while HEK293 cells were transiently transfected with vehicle or constitutively active mutant β-catenin constructs for 24 h and the medium was refreshed (C) prior to ionomycin treatment for 24 h. Lane 1 in (C) is the result of mock transfection (pcDNA). Cells were trypsinized for preparing nuclear extracts, which were used for western blot with anti β-catenin antibody. To demonstrate equivalent loading of the lines, PonceauS staining was used. All pictures are representative of two independently repeated experiments. C, control.

indicated concentration for 24 h, and the nuclear extracts were isolated (Figure 3A). β-Catenin and Tcf-4 were coimmunoprecipitated from the nuclear extracts by using an anti-Tcf-4 antibody. Figure 3A shows that HCT116 cells have substantial levels of the β-catenin–Tcf-4 complex in the nucleus. However, ionomycin decreased the level of β-catenin–Tcf-4 complex dose dependently. Therefore, we concluded that ionomycin inhibits β-catenin/Tcf signaling by decreasing the amount of β-catenin–Tcf complex.

Ionomycin reduced the level of nuclear β-catenin proteins

We considered the possibility that the reduced level of nuclear β-catenin proteins due to ionomycin might result in a reduced association of β-catenin with Tcf-4 leading to the suppressed β-catenin/Tcf signaling. Also, it is well known that Tcf signaling-activation results from the accumulation of nuclear β-catenin (27,28). To investigate this possibility, we performed western blot with the nuclear extracts. HCT116 cells were seeded for 1 day and were treated with ionomycin at the indicated concentrations for 24 h. After 24 h, cells were trypsinized and nuclear extracts were prepared for immunoblotting. Figure 3B shows that substantial levels of nuclear β-catenin products exist in HCT116 cells. However, ionomycin decreased the amount of nuclear β-catenin proteins dose-dependently. Unlike colon cancer cells, low endogenous amounts of β-catenin protein were detected in HEK293 cells (Figure 3C, lane 1), so constitutively active mutant β-catenin gene was transfected into HEK293 cells (Figure 3C). As a result, the level of β-catenin products in the nucleus was elevated considerably by gene transfection (Figure 3C, lane 2). However, like HCT116 cells, this elevated level of nuclear β-catenin products is decreased by ionomycin in a concentration-dependent manner. These results indicate that ionomycin strongly decrease the level of nuclear β-catenin proteins and this leads to a reduced association of β-catenin with Tcf-4 in the nucleus.

Ionomycin disrupts the binding of Tcf complexes to its DNA response element

The inhibitory effect of ionomycin on the transcriptional activity of β-catenin/Tcf might occur by disrupting the binding of β-catenin/Tcf to DNA, so we considered the possibility that the binding activity of Tcf-4 complexes to Tcf response elements (TRE) is decreased by inhibitor. Nuclear extracts derived from 24 h ionomycin treated cells were analyzed by EMSA for their ability in association with a ^{32}P-labeled oligonucleotide containing TRE (Figure 4A). HCT116 cells demonstrate a substantial binding activity of Tcf complexes to TRE (lane 1). However, binding of the Tcf complexes to their specific binding element was decreased by ionomycin. The inhibitory effect of ionomycin against the association of Tcf complexes with DNA was dose dependent (lanes 2–4). To confirm that the upper bands are specific for Tcf binding, we used a ^{32}P unlabeled probe. These bindings could be completely eliminated by adding a 100-fold excess of the ^{32}P unlabeled probe (lanes 5–7). Moreover, as the amount of unlabeled probe incubated with DMSO-treated nuclear extracts (3 μg) increases, the intensity of the bands gets

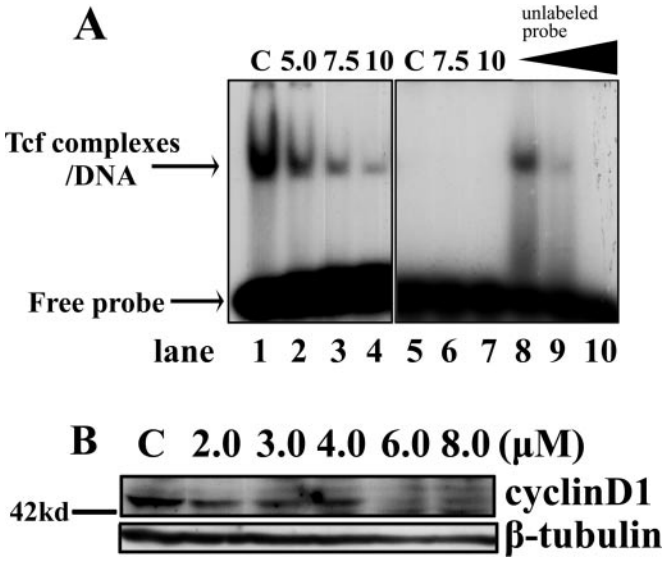

Fig. 4. The binding of Tcf complexes to DNA is decreased by inhibitor, which downregulates the cyclinD1 protein. (**A**) HCT116 cells were treated with ionomycin for 24 h and nuclear extracts were isolated. Nuclear extracts were isolated from cells treated with DMSO (lanes 1, 5 and 8–10) or ionomycin at the indicated concentrations (lanes 2–4 and 6–7). EMSA was performed with 2 μg nuclear extracts from treated or untreated cells. Lanes 1–4 are the results of binding to a ^{32}P-labeled optimal Tcf-binding region. Lanes 5–7 show that 100-fold excess of unlabeled optimal Tcf-binding region used as a competitor prevents binding of the Tcf complexes to the Tcf-binding region. The results of lanes 8–10 were obtained by increasing the amounts of unlabeled oligonucleotide gradually. (**B**) HCT116 cells were treated with ionomycin (2, 3, 4, 6, 8 μM) for 24 h, and trypsinized to isolate cellular extracts and we performed western blot to study the change of cyclinD1 protein level due to ionomycin. To demonstrate equivalent loading of the lines, anti-β-tubulin was used. C, control. All pictures are representative of two independently repeated experiments.

weaker (lanes 8–10). Therefore, we were able to conclude that the upper bands are specific for Tcf binding. Together, we could summarize that the suppressed β-catenin/Tcf signaling could be ascribed to a reduction of binding activity of the Tcf complexes to TRE.

Ionomycin downregulates the expression of β-catenin/Tcf-dependent gene

We investigated whether ionomycin affects the expression of β-catenin/Tcf target gene. It is well known that cyclinD1 is a β-catenin target gene (9). Therefore, we hypothesized that ionomycin would downregulate the cyclinD1 gene expression by inhibiting β-catenin/Tcf signaling. As we expected, cyclinD1 proteins were downregulated by ionomycin (Figure 4B). These results indicate that the functioning of β-catenin as a transcription activator is rendered inoperative by inhibitor.

Discussion

The fact that ionomycin acts as a chemopreventive agent against several types of tumor cells has been reported by some papers (29,30). Here, we tested the effect of ionomycin on the transcriptional activity of β-catenin/Tcf in colon cancer cells. Amongst all human cancers, the molecular pathogenesis of colorectal adenocarcinoma has been one of the most extensively studied. In colon cancer, the elevated β-catenin/Tcf signaling is an important event. Mutations in the regulatory region of β-catenin or the loss of APC function have been identified in human colon cancers (7,23). The β-catenin gene maps to chromosome 3p22, a region of the human genome frequently altered in colon cancers (31). The fact that β-catenin is overexpressed in colon cancer suggests that its levels are no longer regulated by a mutated APC. Indeed, reintroduction of WT APC results in decreased β-catenin levels (32). We hypothesized that the antitumor effects of ionomycin are mediated by its ability to downregulate β-catenin/Tcf signaling, which is proven to be important in colon cancer. Therefore, this study highlights the potential role of ionomycin in the repression of β-catenin/Tcf signaling and its inhibitory mechanism. The studies on the inhibitory agent against β-catenin/Tcf signaling in cancer cell lines have been performed (33–37). As the importance of β-catenin as a cause of tumorigenesis increases, many more studies on the β-catenin inhibitor and its inhibitory mechanism are being conducted. Our data on luciferase activity show that β-catenin/Tcf-driven transcription was suppressed strongly by ionomycin in HCT116 colon cancer cells dose-dependently (Figure 1). Next, we focused on the inhibitory mechanism. Ionomycin also inhibited the constitutively active mutant β-catenin/Tcf signaling in HEK293 cells. In addition, the cellular β-catenin proteins were not degraded by ionomycin treatment. Taken together, these results strongly suggest that the inhibitory mechanism of ionomycin is not related to the upstream regulators of the β-catenin/Tcf pathway but to β-catenin itself or to the downstream components (Figure 2). To investigate the mechanism more precisely, we performed immunoprecipitation. It is well known that the binding of β-catenin to Tcf-4 induces a significant increase in Tcf transcriptional activity (38). However, ionomycin suppressed the association of β-catenin with Tcf-4 in a concentration-dependent manner (Figure 3A). To know the reason why the association between β-catenin and Tcf was suppressed, western blot with nuclear extracts was performed. Here, we hypothesized that ionomycin decreased the amount of nuclear β-catenin proteins which would lead to the reduced binding of β-catenin to Tcf, exemplified by IP data. As we know, β-catenin is ubiquitous and moves freely in a cell. It contributes to the cell–cell adhesion in the membrane, and functions as a transcriptional activator in the nucleus. Dihlmann *et al.* (33,39) and Nath *et al.* (34) reported that the subcellular localization of β-catenin protein was not affected by NSAID and NO-donating aspirin, inhibitors against β-catenin/Tcf signaling. That is, the amount of nuclear β-catenin proteins remains unchanged by NSAID and NO-donating aspirin. We conducted the same experiments with ionomycin (Figure 3B and C). Interestingly, the amount of nuclear β-catenin proteins were decreased significantly by ionomycin in HCT116 and HEK293 cells transiently transfected with constitutively active mutant β-catenin gene. From these data, we were able to conclude that the reduction of nuclear β-catenin proteins results in a decreased association of β-catenin with Tcf-4. To transcript target genes, transcription factors, including β-catenin/Tcf, must bind to consensus DNA, so we asked whether ionomycin affects the DNA-binding properties of the Tcf-4 complexes. Figure 4A shows that the binding of Tcf complexes to consensus DNA is blocked completely by ionomycin in HCT116 cells. Eventually, we concluded that the blockage of the binding of Tcf complexes to consensus DNA by ionomycin, together with reduced nuclear β-catenin proteins, is the main factor in the suppression of the β-catenin/Tcf signaling.

Studies in recent years have suggested that β-catenin accumulation in the nucleus has been implicated in tumorigenesis in a wide variety of human cancers (4,27,28). These studies suggest a corollary: that the blocking of nuclear accumulation of β-catenin suppresses the tumorigenesis. This paper reveals the molecular mechanism underlying the anti-tumor effect of ionomycin from the perspective β-catenin/Tcf signaling. Given its function in inhibiting β-catenin/Tcf signaling, ionomycin may be of interest as a chemotherapeutic agent against tumorigenesis. In further studies, we will focus on the way in which nuclear β-catenin proteins were decreased in colon cancer cells by ionomycin.

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Conflict of Interest Statement: None declared.

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