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Advances in Brief

Differential Formation of β-Catenin/Lymphoid Enhancer Factor-1 DNA Binding Complex Induced by Nitric Oxide in Mouse Colonic Epithelial Cells Differing in Adenomatous Polyposis Coli (*Apc*) Genotype

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Abstract

Increased cytoplasmic β-catenin levels and the associated nuclear β-catenin/T-cell factor (Tcf)-lymphoid enhancer factor (LEF) complex formation have been frequently found in colon cancer. In this context, overproduction of nitric oxide (NO) attributable to inflammatory stimuli in diseases such as ulcerative colitis and Crohn's disease may contribute to colonic carcinogenesis. Therefore, we examined the modulation by NO of cytoplasmic β-catenin levels and the formation of the nuclear β-catenin/LEF-1 DNA binding complex in conditionally immortalized mouse colonic epithelial cells that differed in adenomatous polyposis coli (*Apc*) genotype, namely young adult mouse colon (YAMC; $Apc^{+/+}$) and immortal mouse colon epithelium (IMCE; $Apc^{Min/+}$). Unlike most colon cancer cell lines, this pair of cell lines has either nondetectable or low basal level of β-catenin when they are cultured under nonpermissive and nonproliferative conditions. Using electrophoretic mobility shift assays, we found that NO-releasing agents (E)-methyl-2-[(E)-hydroxyimino]-5-nitro-6-methoxy-3-hexeneamide and *S*-nitroso-*N*-acetylpenicillamine greatly enhanced the formation of β-catenin/LEF-1 DNA binding complex in a concentration- and time-dependent fashion in YAMC and IMCE cells. Significantly, IMCE cells showed a markedly greater amount of nuclear β-catenin/LEF-1 DNA binding complex in response to NO. Super shift by anti-β-catenin antibody confirmed the presence of β-catenin in the complex. Western blot analysis of the soluble cytoplasmic fractions demonstrated that these NO donors caused differential accumulation of cytoplasmic β-catenin in YAMC and IMCE. In conclusion, this study indicates that the defective β-catenin degradation machinery attributable to $Apc^{Min/+}$ mutation in IMCE cells not only affects basal levels but also contributes to NO-induced dysregulation of cytoplasmic β-catenin and nuclear β-catenin/LEF-1 DNA binding complex formation.

Introduction

The role of the *Apc*[3] gene in colonic carcinogenesis and its link with the downstream transcription regulator β-catenin/Tcf-LEF complexes has been recently established (1–3). Although genes responsive to the β-catenin/Tcf-LEF transcription pathway have not been fully identified, an increase in cytoplasmic β-catenin levels and subsequent β-catenin/Tcf-LEF complex formation are believed to be important events in the early stage of colonic carcinogenesis (4, 5). There is also increasing evidence implicating NO in ulcerative colitis and Crohn's disease, conditions known to predispose patients to colon cancer (6, 7). Overexpression of inducible NOS II has been frequently detected in colonic tissues from these patients (6, 7), and NOS II mRNA and protein are overexpressed in colonic adenomas compared with normal tissues (8).

We have recently reported that β-catenin/LEF-1 DNA binding complex may play a role mediating the overexpression of prostaglandin endoperoxide H synthase-2 induced by NO (9). To further explore the connection between *Apc*, β-catenin, and NO, we examined the formation of nuclear β-catenin/LEF-1 DNA binding complex as well as the increase of cytoplasmic β-catenin in response to NO-releasing drugs in two nontransformed and nontumorigenic murine colonic epithelial cells with distinct *Apc* genotypes. In this pair of cell lines with similar genetic backgrounds, one carries the $Apc^{Min/+}$ mutation and is associated with defective degradation and the greater accumulation of β-catenin. These conditionally immortal cells are designated YAMC ($Apc^{+/+}$) derived from a SV40LT antigen parental mouse and IMCE ($Apc^{Min/+}$) derived from the F1 hybrids resulting from the mating of $Apc^{Min/+}$ and SV40LT antigen transgenic mice. Because both YAMC and IMCE express the heat-labile SV40LT antigen that allows them to proliferate at 33°C, they revert to a nontransformed phenotype at the restrictive temperature of 39°C, at which the proliferation of these cells ceases (10–12). The temperature-sensitive SV40LT mutant antigen becomes inactivated and nonfunctional when cells are transferred to 39°C for 72 h before each experiment. The genotype and expression of APC protein have been confirmed in these cells by allele-specific PCR and by Western immunoblotting, respectively (10). The epithelial nature of these cells was demonstrated by staining with antikeratin antisera (11). Furthermore, these cells have been used to demonstrate that the $Apc^{Min/+}$ mutation in IMCE cells can cooperate with stably transduced oncogenic *ras* to produce the transformed, tumorigenic phenotype (*e.g.*, growth in soft agar; tumor formation in athymic mice; 13). All of the experiments in this study were carried out under nonpermissive and nonproliferative conditions.

Data from the present study suggest that NO may increase cytoplasmic β-catenin levels and, therefore, enhance the differential formation of β-catenin/LEF-1 DNA binding complexes in YAMC and IMCE cells under these conditions. Significantly, the increased formation of nuclear β-catenin/LEF-1 DNA binding complex via accumulation of free β-catenin in the cytoplasm is differentially expressed based on their respective *Apc* genotypes.

Materials and Methods

Materials. Anti-β-catenin, anti-E-cadherin and antimouse IgG horseradish peroxidase-conjugated monoclonal antibodies were purchased from Transduction Laboratories (Lexington, KY). Anti-β-catenin polyclonal antibody was from Sigma (St. Louis, MO). Antiactin monoclonal antibody was from Boehringer Mannheim (Indianapolis, IN). For cell culture, the following products were used and purchased from their respective sources: RPMI 1640 and mouse IFN-γ from

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[3] The abbreviations used are: Apc, adenomatous polyposis coli; NO, nitric oxide; NOS II, NO synthase II; LEF, lymphoid enhancer factor; Tcf, T-cell factor; GSK-3β, glycogen synthase kinase 3β; YAMC, young adult mouse colon; IMCE, immortal mouse colon epithelium; NOR-1, (E)-methyl-2-[(E)-hydroxyimino]-5-nitro-6-methoxy-3-hexeneamide; SNAP, *S*-nitroso-*N*-acetylpenicillamine; EMSA, electrophoretic mobility shift assay; ECM, extracellular matrix.

Life Technologies, Inc. (Grand Island, NY); neonatal calf serum from Gemini Bio-Products (Calabasas, CA), ITS+ from Collaborative Biomedical (Bedford, MA). The NO donors NOR-1 and SNAP were purchased from Calbiochem (San Diego, CA). All other chemicals and reagents were purchased from Sigma unless indicated otherwise.

Cell Culture. Experiments were carried out using the conditionally immortalized murine colonic epithelial cell (9–12). Both YAMC and IMCE cells express the heat-labile SV40 large T antigen with an IFN-γ-inducible promoter. The temperature-sensitive SV40 large T antigen is inactivated and becomes nonfunctional if the temperature is changed to 39°C. All of the cells were grown in 75-cm^2 culture flasks coated with type I collagen (5 μg/cm^2) in RPMI 1640 supplemented with 5% neonatal calf serum, ITS+ (6.25 μg/ml insulin, 6.25 μg/ml transferrin, 6.25 ng/ml selenious acid, 5.35 mg/ml linoleic acid, and 1.25 mg/ml BSA), 5 IU/ml murine IFN-γ, 100,000 IU/liter penicillin, and 100 mg/liter streptomycin. They were cultured under transforming (permissive) conditions in a 33°C incubator with 5% CO_2. On attaining confluency, all of the cells were then transferred to nontransforming (nonpermissive) conditions at 39°C in serum-free and IFN-γ-free media for 72 h before each experiment.

Preparation of Nuclear Extracts and EMSAs. Nuclear extracts were prepared from YAMC and IMCE cells according to the method described previously (9). Briefly, cells were rinsed once with cold PBS followed by trypsinization. After centrifugation at 1000 × *g* for 5 min, they were resuspended in 5-pellet volumes of hypotonic buffer containing 0.2 mM phenylmethylsulfonyl fluoride and 0.5 mM DTT. They were then chilled on ice for 10 min followed by lysis with a PT 3000 Polytron (Brinkmann, Littau, Switzerland) for 30 s at 14,000 rpm and centrifuged at 4000 × *g* for 15 min. The pellet was resuspended in 0.5-pellet volumes of low-salt buffer. An equal volume of high-salt buffer was added drop-wise to the gently stirred suspension. The nuclear extracts were subjected to centrifugation at 16,000 × *g* for 30 min followed by overnight dialysis. Nuclear protein (5 μg) was added to a 20-μl reaction mix containing 300 ng of poly(dI·dC); binding buffer [10 mM HEPES (pH 7.6), 60 μM KCl, 1 mM EDTA, 1 mM DTT, and 12% glycerol]; with or without double-stranded mouse LEF-1 oligonucleotide (Life Technologies, Inc.), CACCCTTTGAAGCTC with 5′ overhang, as a specific competitor. Samples were incubated on ice for 10 min. Then LEF-1 oligonucleotide, radio-labeled using T4 kinase (Life Technologies, Inc.) and [γ-^{32}P]ATP (NEN, Boston, MA), was added at 1.5–2 × 10^4 cpm per reaction and incubated at room temperature for 30 min. DNA loading dye (Quality Biological, Gaithersburg, MD) was added to stop the reaction. Samples were run on a 4% polyacrylamide (37.5:1; Protogel; National Diagnostics, Atlanta, GA) gel at 189V for 2.5 h in 0.5 × Tris-borate EDTA running buffer. Gels were dried and exposed to XAR-5 film (Kodak). For super-shift studies, 3–5 μg of nuclear lysate were mixed in a 20-μl reaction mixture as described for EMSA, and that mixture was incubated on ice for 10 min. Antibodies, 12 μg of polyclonal anti-β-catenin and 500 ng of monoclonal anti-E-cadherin, or rabbit IgG, were then added to the respective reaction tubes. Reactions were incubated on ice for 15 min. γ-^{32}P-labeled murine LEF-1 oligonucleotide probe was added at 1.5–2 × 10^4 cpm per reaction and incubated at room temperature for 30 min.

Western Blot Analysis. Briefly, cells were washed twice with cold PBS and were harvested either under denaturing conditions by scraping in boiling 2× Laemmli sample buffer (Bio-Rad, Hercules, CA) or under nondenaturing conditions by using a radioimmunoprecipitation assay buffer set (RIPA; Boehringer Mannheim). For total cell lysates under denaturing conditions, samples were heated at boiling temperature for an additional 5 min in Laemmli sample buffer. Homogenates were then prepared by sonication (1 min each; Sonifier; Branson, Danbury, CT). After centrifugation at 2000 × *g* for 5 min, the supernatants were used as the protein source. To make protein preparations that would contain only soluble cytoplasmic fractions, cells were lysed in radioimmunoprecipitation assay buffer under nondenaturing conditions at 4°C. They were then incubated on ice for 15 min on a shaker. The homogenized soluble supernatants were prepared by centrifugation at 100,000 × *g* for 30 min at 4°C. The protein concentration was determined by the bicinchoninic acid method (Pierce, Rockford, IL). Electrophoresis samples were prepared by mixing the respective protein preparations with 2× Laemmli sample buffer. To each well of SDS-polyacrylamide gel, 15–30 μl of cell lysate (about 15–30 μg of total protein) were applied and electrophoresed in 0.75-mm thick 7.5% Tris-Glycine gel (Bio-Rad, Hercules, CA). Proteins were transferred to nitrocellulose membrane using a semidry blotter (Bio-Rad) at 15V for 30 min. Blots were then probed with the respective primary antibodies at the manufacturer's suggested dilution followed by a secondary antimouse IgG antibody conjugated to horseradish peroxidase (1:2000). Detection was by an ECL kit (Amersham, Arlington Heights, IL). Blots were routinely stripped by Encore Blot Stripping Kit (Novus Molecular, Inc., San Diego, CA) and reprobed with anti-actin monoclonal antibody to serve as loading controls.

Results

Differential Formation of DNA/β-Catenin/LEF-1 Complex in YAMC and IMCE Cells Induced by NO. In contrast to cell lines derived from colon tumors that often show constitutively high levels of cytoplasmic β-catenin and nuclear β-catenin/Tcf-LEF activity, the nontransformed and nontumorigenic colonic epithelial cells express little (YAMC) or relatively low (IMCE) levels of β-catenin/LEF-1 DNA binding complex when cultured under nontransforming conditions. As shown in Fig. 1, IMCE cells had a slightly higher basal expression of this complex than did YAMC under these conditions. This is consistent with the defective β-catenin degradation machinery attributable to the *Apc*$^{Min/+}$ mutation in IMCE cells. It suggests that some β-catenin accumulates in resting IMCE cells instead of being degraded rapidly as occurs in YAMC, and, consequently, more β-catenin/LEF-1 DNA binding complex formed in the nucleus. As we reported recently (9), the formation of the β-catenin/LEF-1 DNA binding complex was greatly enhanced by the two NO donors, NOR-1 and SNAP, which both release NO spontaneously when dissolved in aqueous solution and do not share any structural similarity. The amount of β-catenin/LEF-1 DNA binding complex induced by either NOR-1 or SNAP appeared to be concentration- (Fig. 1*A*) and time-dependent (Fig. 1, *B* and *C*), which indicated the specificity of the NO effects. In all of the EMSA experiments, an excess amount of unlabeled LEF-1 probe effectively competed away the β-catenin/LEF-1 DNA binding complex, which demonstrated the participation of LEF-1 in the complex and indicated its specificity. More importantly, the difference in the β-catenin/LEF-1 DNA binding complex formation between YAMC and IMCE cells widened markedly in response to NO, which indicated that APC may be involved.

Confirmation of β-Catenin in NO-induced DNA/β-Catenin/LEF-1 Complex by Super-Shifting. The participation of β-catenin in the DNA binding complex was confirmed by a series of super-shift assays using anti-β-catenin antibody (Fig. 2). Nonspecific antibodies, namely anti-E-cadherin antibody and rabbit preimmune IgG, were used as controls in the super-shift assays and did not cause any change in the migration pattern. As shown earlier, unlabeled LEF-1 probe effectively competed away the β-catenin/LEF-1 DNA binding complex (Fig. 2). This study directly demonstrates the presence of β-catenin in the DNA binding complex in conjunction with LEF-1. More interestingly, the difference in β-catenin/LEF DNA binding complex formation between YAMC and IMCE was evident and was markedly amplified by NO treatment. Both NOR-1 and SNAP showed similar effects on the induction of this complex formation, although NOR-1 appeared to be more potent than SNAP (Fig. 2*B*). Because these two agents have different kinetics in terms of NO release, they may, therefore, have different patterns of concentration- and time-dependence on the complex formation as suggested in Fig. 2*B*. Nevertheless, β-catenin was detected by super-shift in the complex formed in response to either drug.

NO Causes Differential Accumulation of Free Cytoplasmic β-Catenin but not Total β-Catenin in YAMC and IMCE Cells. The aforementioned results suggest that NO produced an increase in the heterodimeric transcriptional factor, β-catenin/LEF-1. There are several possibilities to explain this finding, including the NO-augmentation of complex formation and/or increased translocation of the complex into the nucleus. The most likely explanation, however, is that NO caused an accumulation of cytoplasmic β-catenin available for transport into the nucleus. The localization of β-catenin is normally associated with the transmembrane glycoprotein E-cadherin. β-catenin remains at very low levels in the normal resting cells because of its constant degradation by APC and GSK-3β in conjunction with Axin (14–16). β-Catenin is

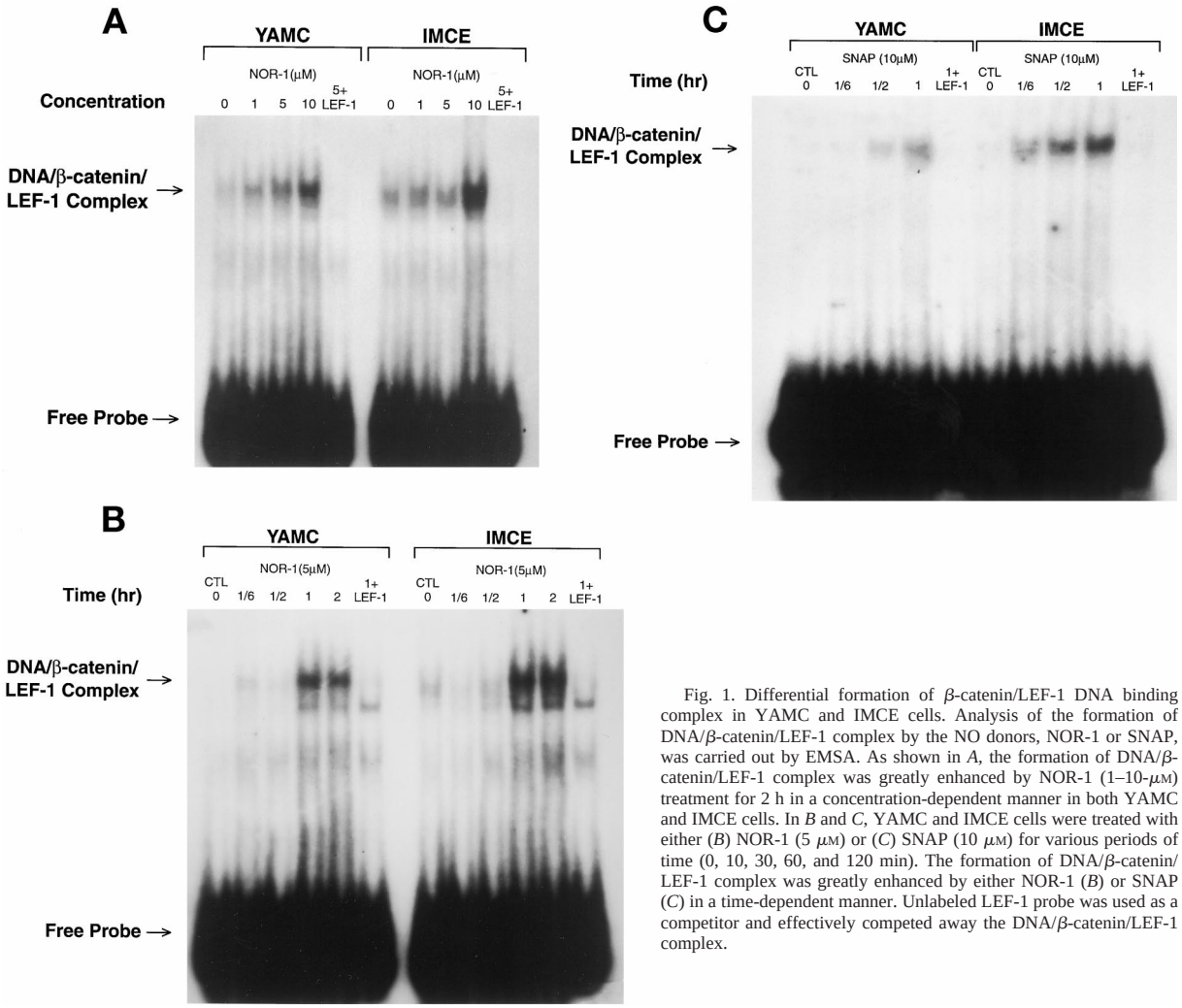

Fig. 1. Differential formation of β-catenin/LEF-1 DNA binding complex in YAMC and IMCE cells. Analysis of the formation of DNA/β-catenin/LEF-1 complex by the NO donors, NOR-1 or SNAP, was carried out by EMSA. As shown in *A*, the formation of DNA/β-catenin/LEF-1 complex was greatly enhanced by NOR-1 (1–10-μM) treatment for 2 h in a concentration-dependent manner in both YAMC and IMCE cells. In *B* and *C*, YAMC and IMCE cells were treated with either (*B*) NOR-1 (5 μM) or (*C*) SNAP (10 μM) for various periods of time (0, 10, 30, 60, and 120 min). The formation of DNA/β-catenin/LEF-1 complex was greatly enhanced by either NOR-1 (*B*) or SNAP (*C*) in a time-dependent manner. Unlabeled LEF-1 probe was used as a competitor and effectively competed away the DNA/β-catenin/LEF-1 complex.

primarily localized in the membrane in these nontransformed colonic epithelial cells presumably bound to the transmembrane adhesion molecule E-cadherin (9). We explored the basis for the increased DNA binding activity of the nuclear β-catenin/LEF-1 complex. The amount of free β-catenin in the soluble cytoplasmic fractions showed dramatic increases from nondetectable (YAMC) or low (IMCE) levels to greatly increased levels after treatment with the NO donors (Fig. 3*A*). No E-cadherin was detected in these soluble cytoplasmic fractions (data not shown), which suggests that the assayed β-catenin was no longer bound to E-cadherin. In contrast, the amounts of total β-catenin in whole-cell lysates were examined and did not change after various treatments with the NO donors, NOR- 1 (5 μM) and SNAP (10 μM; see Fig. 3*B*). Although this finding does not conclusively reveal the mechanism responsible for the increase in the free cytoplasmic pool of β-catenin, it implies that NO may facilitate the nuclear β-catenin/LEF-1 complex formation by increasing the amount of free cytoplasmic β-catenin available for its translocation into the nucleus. Because these nontransformed and nontumorigenic cells have little free β-catenin in the cytoplasm, as demonstrated in Fig. 3*A*, it is likely that NO caused the disruption of the association between β-catenin and membrane-bound E-cadherin, thereby releasing it into the cytoplasm. As we know, YAMC and IMCE cells differ in β-catenin degradation because of their genetic difference in *Apc*. We found that IMCE cells had much higher amounts of free β-catenin accumulated in the cytoplasm in response to NO (Fig. 3*A*). Importantly, the total amount of β-catenin was not different between the two cell lines nor did either amount change after NO exposure (Fig. 3*B*). These data suggest that the accumulation of β-catenin in cytoplasm was probably the net result of dissociation from the membrane and the degradation by APC, GSK-3β, and Axin. The NO-stimulated turnover of β-catenin was further examined in a time course study (Fig. 4). NOR-1 treatment, as early as 10 min, markedly increased free β-catenin accumulation in the cytoplasm of both YAMC and IMCE cells, a finding that supports the dissociation of β-catenin from the membrane and militates against alteration of *de novo* synthetic mechanisms. Corroborating the results shown in Fig. 3, we found that cytoplasmic levels of β-catenin reached much higher levels in IMCE cells than in YAMC cells. It seems likely that the defective degradation apparatus for β-catenin in IMCE cells results in a higher level of β-catenin accumulation once β-catenin is freed from membrane. The mechanism by which NO causes the dissociation of β-catenin from membrane-bound E-cadherin remains unknown but is currently under vigorous investigation in this laboratory.

Discussion

Our demonstration that NO is involved in the accumulation of cytoplasmic β-catenin and in the formation of the β-catenin/LEF-1 DNA binding complex in the conditionally immortal murine colonic epithelial cells may help elucidate the early molecular events during colorectal carcinogenesis. Because of the relatively low cytoplasmic β-catenin when cultured under nonpermissive conditions, these nontransformed and nontumorigenic cells provide an ideal cell system in which to

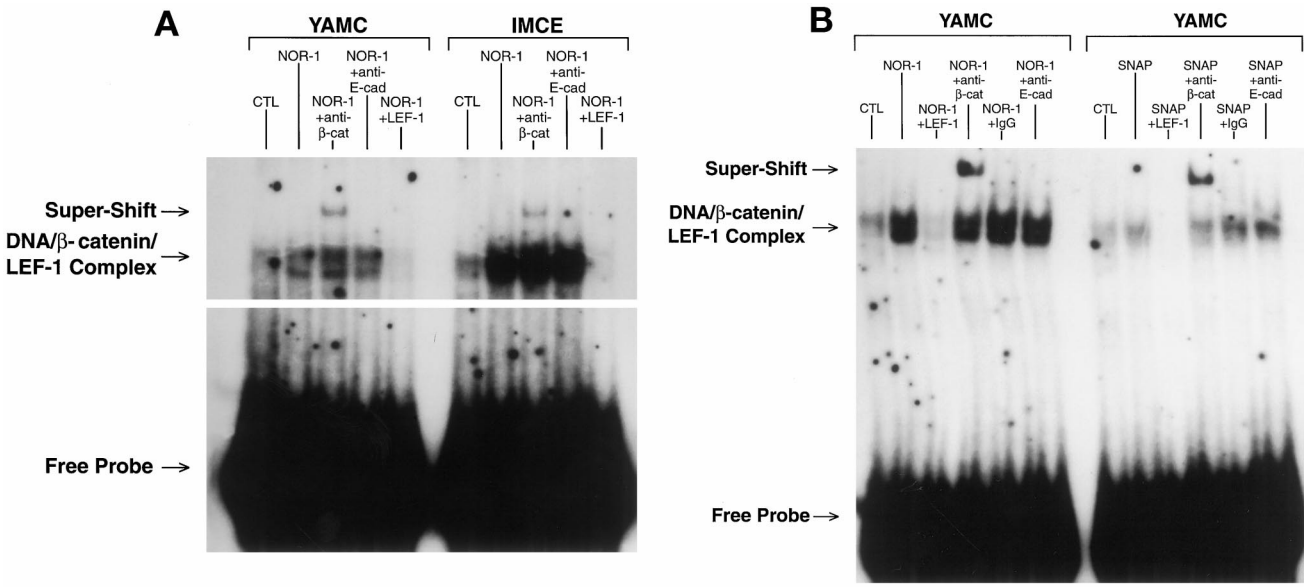

Fig. 2. Super-shifting of the DNA/β-catenin/LEF-1 complex by anti-β-catenin antibody. As shown in *A* and *B*, the suggested participation of β-catenin in the DNA binding complex was confirmed by super-shift assays using a polyclonal anti-β-catenin antibody. YAMC and IMCE cells were first treated with either NOR-1 (5 μM) or SNAP (10 μM) for 60 min before nuclear extracts were harvested. Nonspecific anti-E-cadherin antibody and rabbit IgG were used as controls in the super-shift assays and did not cause any super-shifting of the complex. Unlabeled LEF-1 probe was used as a competitor and effectively competed away the DNA/β-catenin/LEF-1 complex.

examine an early event in colorectal carcinogenesis, namely the dissociation and regulation of membrane-bound as well as cytoplasmic β-catenin in response to environmental stimuli. Recent studies have emphasized the importance of *Apc* mutations and their effects on β-catenin in the early phase of colorectal tumor development (1–5). APC, along with interacting proteins GSK-3β and Axin, actively degrades β-catenin and maintains cytoplasmic β-catenin at very low levels (14–16). Direct or indirect modulation of the degradation process that results in the accumulation of cytoplasmic β-catenin leads to the formation of a transcriptionally active β-catenin/Tcf-LEF complex. The integrated regulation of these processes in normal physiology is not well understood, but their disruption has been associated with the transformation of normal colonic epithelium to adenomas and adenocarcinomas (2–4).

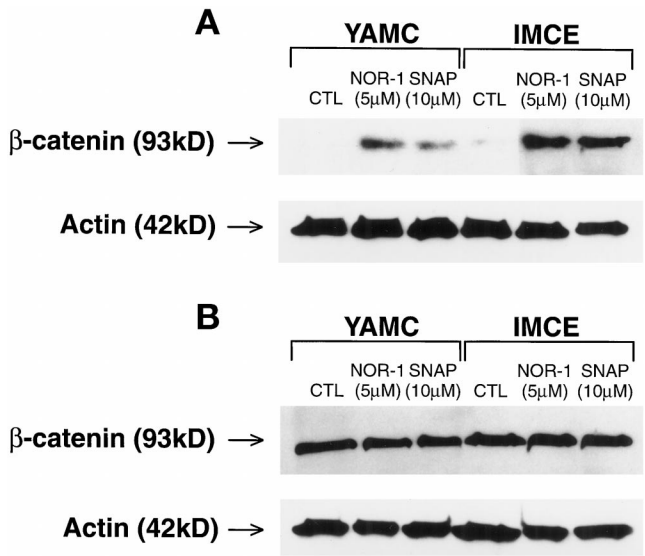

Fig. 3. Analysis by Western blot of the redistribution of β-catenin into the soluble cytoplasmic fraction in response to the NO donors, NOR-1 or SNAP. YAMC and IMCE cells were stimulated with either NOR-1 (5 μM) or SNAP (10 μM) for 2 h. Cells were then harvested and were fractionated into either soluble cytoplasmic preparations or total cell lysates. In *A*, β-catenin was detected only in the soluble cytoplasmic fractions from cells treated for 2 h with either NOR-1 or SNAP, but none (*YAMC*) or little (*IMCE*) was found in the untreated cells. Soluble fractions (30 μg) were applied to each lane of the precast Bio-Rad 7.5% Tris-HCl gels. β-catenin was detected by an anti-β-catenin monoclonal antibody. The same blot was then stripped and reprobed for actin as a loading control. In *B*, β-catenin was detected by Western blot in the total-cell lysates and remained constant in both YAMC and IMCE cells after exposure to either NOR-1 or SNAP for 2 h. Total-cell lysates (15 μg) were applied to each lane of the precast Bio-Rad 7.5% Tris-HCl gels. The same blot was then stripped and reprobed for actin as a loading control.

Although free β-catenin is kept at a very low level in the cytoplasm and nucleus by APC mediated degradation, an abundant amount of β-catenin is normally associated with the transmembrane glycoprotein E-cadherin. The E-cadherin-bound β-catenin is also called the "insoluble" β-catenin in that it is not free to interact with other proteins in the cytoplasm. It has been reported, however, that the dissociation of β-catenin from E-cadherin can increase free soluble β-catenin in the cytoplasm, and, importantly, the association of β-catenin and E-cadherin can be affected by a variety of signals directly or indirectly (17–19). It is now known that activation of the membrane-bound metalloprotease can cause the loss of β-catenin from E-cadherin and cell-cell contacts and can, thereby, activate the β-catenin-mediated intracellular signaling pathway (20). The metalloprotease-induced dissociation and intracellular translocation of β-catenin can be facilitated by intracellular calcium influx (20). Interestingly, it is also documented that NO can activate this membrane-bound metalloprotease (21, 22). NO has been found to increase intracellular calcium as well (23–25). Our data, as demonstrated in Fig. 3 and 4 in particular, suggest that NO may be able to facilitate such dissociation and thereby increase free β-catenin in the cytoplasm and nucleus. The morphological changes of NOR-1 treated cells, namely the enlargement as well as the swelling and blebbing of the cells (9), could be attributable to the dissociation of the β-catenin and E-cadherin complex at the membrane and the subsequent disruption of the adherens junctions at the cell-cell border (17–19). Our demonstration that NO may cause the possible dissociation of membrane-bound β-catenin and the formation of nuclear β-catenin/LEF-1 DNA binding complex may be important events in the early stage of colonic carcinogenesis. We are currently investigating whether the effect of NO on intracellular calcium mobilization and activation of matrix metalloprotease is causally related to its effect on the dissociation of β-catenin from membrane-bound E-cadherins.

Another possible mechanism for NO to exert its effect on cytoplasmic β-catenin accumulation is through the disruption of the β-catenin degradation apparatus, namely the connections among APC, GSK-3β, Axin,

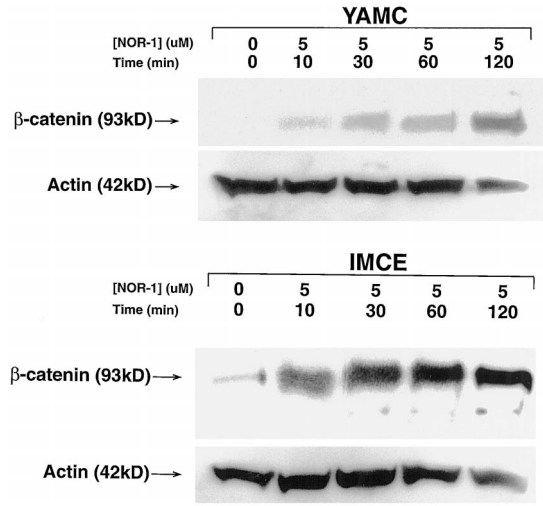

Fig. 4. Time course analysis of the differential cytoplasmic β-catenin accumulation in response to NO in YAMC and IMCE cells. YAMC and IMCE cells were treated with NOR-1 (5 μM) for various periods of time (*0*, *10*, *30*, *60*, and *120* min). The soluble cytoplasmic fractions were prepared and were analyzed by Western blot. Cytoplasmic β-catenin accumulation in response to NOR-1 appeared to be time-dependent and differential in YAMC and IMCE cells. Soluble fractions (30 μg) were applied to each lane of the precast Bio-Rad 7.5% Tris-HCl gels. β-catenin was detected by an anti-β-catenin monoclonal antibody. The same blots were then stripped and probed for actin as a loading control.

and β-catenin, the "tetrameric" interactions. For this degradation process to be effective and efficient, regulated interactions are essential among the proteins involved. For example, the recruitment of GSK-3β and β-catenin by Axin must take place prior to the phosphorylation of β-catenin, which is crucial for its subsequent degradation by APC (16). This complex interaction presumably is redox-sensitive and, therefore, could be affected by NO. In addition, the mechanism by which β-catenin is transported from the cytoplasm to nucleus remains unclear. In the context of nuclear protein trafficking, it is plausible that there is a specific transporter protein that facilitates the crossing of the nuclear membrane by β-catenin. Whether NO plays any role in this translocation remains an intriguing question.

Although the exact role of NO in tumor biology is not completely understood, NO is undoubtedly a potent mediator in the processes of inflammation, infection, and cancer. NO and NOS II have been suggested to play important roles in the early events of colonic carcinogenesis (26, 27). Furthermore, NO has been found to have important interactions with ECM metabolism (28, 29). In addition to its effect on matrix-associated metalloprotease mentioned earlier (21), NO, produced by inflammatory cells such as macrophages, has been reported to affect the turnover of ECM of neighboring cells and to influence cellular attachment, migration, and proliferation through its interactions with ECM (28, 29). The finding that NO can trigger the accumulation of free soluble cytoplasmic β-catenin and the formation of β-catenin/LEF-1 DNA binding complex, as presented in this study, supports the hypothesis that NO may be involved in the early stages of colorectal carcinogenesis. This may be especially important when genetic predisposition, such as $Apc^{Min/+}$ mutation, is concurrently present.

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