Regulation of adherens junctions and epithelial paracellular permeability: a novel function for polyamines

Xin Guo, Jaladanka N. Rao, Lan Liu, Tong-Tong Zou, Douglas J. Turner, Barbara L. Bass, and Jian-Ying Wang. Regulation of adherens junctions and epithelial paracellular permeability: a novel function for polyamines. Am J Physiol Cell Physiol 285: C1174–C1187, 2003. First published July 9, 2003; 10.1152/ajpcell.00015.2003.—Maintenance of intestinal mucosal epithelial integrity requires polyamines that are involved in the multiple signaling pathways controlling gene expression and different epithelial cell functions. Integrity of the intestinal epithelial barrier depends on a complex of proteins composing different intercellular junctions, including tight junctions, adherens junctions, and desmosomes. E-cadherin is primarily found at the adherens junctions and plays a critical role in cell-cell adhesions that are fundamental to formation of the intestinal epithelial barrier. The current study determined whether polyamines regulate intestinal epithelial barrier function by altering E-cadherin expression. Depletion of cellular polyamines by α-difluoromethylornithine (DFMO) reduced intracellular free Ca²⁺ concentration ([Ca²⁺]_{cyt}), decreased E-cadherin expression, and increased paracellular permeability in normal intestinal epithelial cells (IEC-6 line). Polyamine depletion did not alter expression of tight junction proteins such as zona occludens (ZO)-1, ZO-2, and junctional adhesion molecule (JAM)-1. Addition of exogenous polyamine spermidine reversed the effects of DFMO on [Ca²⁺]_{cyt} and E-cadherin expression and restored paracellular permeability to near normal. Elevation of [Ca²⁺]_{cyt} by the Ca²⁺ ionophore ionomycin increased E-cadherin expression in polyamine-deficient cells. In contrast, reduction of [Ca²⁺]_{cyt} by polyamine depletion or removal of extracellular Ca²⁺ not only inhibited expression of E-cadherin mRNA but also decreased the half-life of E-cadherin protein. These results indicate that polyamines regulate intestinal epithelial paracellular barrier function by altering E-cadherin expression and that polyamines are essential for E-cadherin expression at least partially through [Ca²⁺]_{cyt}.

ornithine decarboxylase; tight junction; catenins; intracellular calcium; normal intestinal epithelial cells

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plasma membranes together physically and permit or enhance tight junction assembly. In support of this possibility, anti-cadherin antibodies that block the formation of adherens junctions also inhibit the formation of tight junctions, thus resulting in an increase in epithelial paracellular permeability (4, 29, 50).

The natural polyamines, including spermidine, spermine, and their precursor putrescine, are organic cations found in all eukaryotic cells (49). Polyamines have been implicated in a wide variety of biological functions, and regulation of cellular polyamines has been recognized to be the central convergence point for the multiple signaling pathways driving different epithelial cell functions including cell motility (30, 56, 58), the multiple signaling pathways driving different epithelial cell functions including cell motility (30, 56, 58), proliferation (24, 26, 35–37), and apoptosis (25, 44). We (41, 43, 59) recently found that polyamines regulate intracellular free Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_{\text{cyt}}\)) primarily by governing membrane potential (\(E_m\)) through control of voltage-gated K\(^+\) (Kv) channel expression and that elevated [Ca\(^{2+}\)]\(_{\text{cyt}}\) is a major mediator for the stimulation of distinct intestinal epithelial functions after increase in polyamines. Because intestinal epithelial cells do not express L-type voltage-dependent Ca\(^{2+}\) channels (VDCC), polyamines stimulate Kv channel expression, resulting in membrane hyperpolarization, and increase [Ca\(^{2+}\)]\(_{\text{cyt}}\) by enhancing the driving force for Ca\(^{2+}\) influx. Using an in vitro cell migration model mimicking the early cell division-independent stage of mucosal restitution, we (15) further demonstrated that polyamines regulate β-catenin tyrosine phosphorylation through [Ca\(^{2+}\)]\(_{\text{cyt}}\), implicating these compounds in the modulation of cell-cell adhesions and barrier function in the intestinal epithelium.

The aim of current study was to test the hypothesis that polyamines are involved in regulation of intestinal epithelial barrier function by altering E-cadherin expression through Ca\(^{2+}\). First, we examined effects of polyamine depletion on expression and cellular distribution of the adherens junction proteins E-cadherin, β-catenin, and α-catenin and the tight junction proteins zona occludens (ZO)-1, ZO-2, and junctional adhesion molecule (JAM)-1 in normal intestinal epithelial cells (IEC-6 line). Second, we determined whether manipulation of [Ca\(^{2+}\)]\(_{\text{cyt}}\), either increase or decrease, alters E-cadherin expression in the presence or absence of polyamines. Third, we investigated whether the observed changes in E-cadherin expression affect intestinal epithelial paracellular permeability after polyamine depletion.

**MATERIALS AND METHODS**

**Chemicals and supplies.** Disposable culture ware was purchased from Corning Glass Works (Corning, NY). Tissue culture media and dialyzed fetal bovine serum (dFBS) were obtained from Gibco-BRL (Gaithersburg, MD), and biochemicals were from Sigma (St. Louis, MO). The monoclonal antibodies against E-cadherin, β-catenin, and α-catenin were purchased from Transduction Laboratories (Lexington, KY). The affinity-purified rabbit polyclonal antibodies against ZO-1, ZO-2, claudin-2, and claudin-4 were purchased from Zymed Laboratories (San Francisco, CA). The antibody against JAM-1 was obtained from R&D Systems (Minneapolis, MN). α-Difluoromethylornithine (DFMO) was purchased from Ilex Oncology (San Antonio, TX). The 12-mm Transwell filters (0.4-μm pore size, clear polyester) were obtained from Costar (Cambridge, MA). Fluorescein-conjugated goat antimouse and goat anti-rabbit antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). \(^3^H\)-labeled mannitol and \(^3^H\)-labeled inulin were obtained from Amersham Pharmacia Biotech (Piscataway, NJ).

**Cell cultures and general experimental protocols.** The IEC-6 cell line was purchased from the American Type Culture Collection (ATCC) at passage 13. The cell line was derived from normal rat intestine and was developed and characterized by Quaroni et al. (39). IEC-6 cells originated from intestinal crypt cells, as judged by morphological and immunologic criteria. They are nontumorigenic and retain the undifferentiated character of epithelial stem cells. Stock cells were maintained in T-150 flasks in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 5% heat-inactivated FBS, 10 μg/ml insulin, and 50 μg/ml gentamicin sulfate. Flasks were incubated at 37°C in a humidified atmosphere of 90% air-10% CO\(_2\). IEC-6 cells were subcultured once a week at 1:20; medium was changed three times weekly. The cells were restarted from original frozen stock every seven passages. Tests for mycoplasma were routinely negative, and passages 15–20 were used in the experiments.

The stable Cdx2-transfected-IEC-6 cell line was developed and characterized by Suh and Traber (48) and was a kind gift from Dr. Peter G. Traber (University of Pennsylvania, Philadelphia, PA). The expression vector, the LacSwitch System (Stratagene, La Jolla, CA), was used for directing the conditional expression of Cdx2, and isopropyl-β-D-thiogalactopyranoside (IPTG) served as the inducer for gene expression. IEC-6 cells were transfected with pOPRSCdx2 by an electroporation technique and clones resistant to selection medium containing 0.6 mg/ml G418 and 0.3 μg/ml hygromycin B were isolated and screened for Cdx2 expression by Northern blot, RNase protection assays, and electrophoretic mobility shift assay. Stock stable Cdx2-transfected IEC-6 cells were grown in the DMEM used in parental nontransfected IEC-6 cells. Before experiments, cells were grown in DMEM containing 4 mM IPTG for 16 days to induce cell differentiation.

In the first series of studies, we examined the effects of polyamine depletion on expression and cellular distribution of E-cadherin, β-catenin, α-catenin, ZO-1, ZO-2, and JAM-1 in IEC-6 cells. Cells were grown in control cultures or cultures containing 5 mM DFMO or DFMO plus 5 μM spermidine for 4, 6, or 8 days, and the monolayers were washed three times with ice-cold Dulbecco’s PBS (DPBS). Different solutions were added according to the assays to be conducted.

In the second series of studies, we investigated whether manipulation of [Ca\(^{2+}\)]\(_{\text{cyt}}\), either increase or decrease, alters E-cadherin expression in the presence or absence of polyamines. The Ca\(^{2+}\) ionophore ionomycin (1 μM) was used to increase [Ca\(^{2+}\)]\(_{\text{cyt}}\), whereas Ca\(^{2+}\)-free medium was used to decrease [Ca\(^{2+}\)]\(_{\text{cyt}}\). In the Ca\(^{2+}\)-free medium, 1.8 mM CaCl\(_2\) was replaced by 1.8 mM MgCl\(_2\) and an additional 0.1 mM EGTA was added to chelate the residual Ca\(^{2+}\). Free Ca\(^{2+}\) in the Ca\(^{2+}\)-free medium was <0.002 mM. Levels of E-cadherin mRNA and protein were measured at various times after treatment with 1 mM thiononycin or Ca\(^{2+}\)-free medium in normal (without DFMO) and polyamine-deficient (with DFMO) IEC-6 cells.

In the third series of studies, we investigated whether observed changes in E-cadherin expression after polyamine depletion alter paracellular permeability in parental IEC-6
cells and differentiated IEC-Cdx2L1 cells. Permeability was measured by paracellular tracer flux assays, and membrane-impermeable molecules, [14C]mannitol and [3H]inulin, measured by paracellular tracer assays.

Reverse transcription and PCR. Total RNA was isolated by using an RNeasy Mini Kit (Qiagen, Valencia, CA). Equal amounts of total RNA (2 μg) were transcribed to single-strand cDNA with a reverse transcription (RT)-PCR kit (Invitrogen Life Technologies, Carlsbad, CA). Specific primers for E-cadherin, β-catenin, α-catenin, ZO-1, and ZO-2 were designed from the cDNA sequences of the coding regions corresponding to these genes (Table 1). These particular sequences were chosen on the basis of specificity established by previous publications from others (8, 21, 64). RT-PCR was performed as described in our earlier publications (43, 60). To quantify the PCR products (the amounts of mRNA) of E-cadherin, β-catenin, α-catenin, ZO-1, and ZO-2, an invariant mRNA of β-actin was served as tracers. Cell samples were sonicated and centrifuged at 4°C, and the supernatants were collected for immunoprecipitation. Equal amounts of proteins (300 μg) for each sample were incubated with the specific antibody against E-cadherin (2 μg) at 4°C for 3 h, and protein G-PLUS-Agarose was added and incubated overnight at 4°C. The precipitates were washed with ice-cold D-PBS, and the beads were resuspended in SDS sample buffer for subsequent gel electrophoresis (SDS-PAGE, 7.5% ready gel). The gels were dried and exposed to X-ray film with an intensifying screen.

Immunofluorescence staining. The immunofluorescence procedure was carried out according to the method of Vielkind and Wierenga (55) with minor changes (15, 58). Cells were incubated with the primary antibody against E-cadherin, β-catenin, α-catenin, ZO-1, or ZO-2 at 4°C overnight and then incubated with secondary antibody conjugated with FITC for 2 h at room temperature. After the slides were rinsed three times, they were mounted and viewed through a Zeiss confocal microscope (model LSM410). Images were processed with Photoshop software (Adobe, San Jose, CA).

Measurement of [Ca2+]cyt. Details of the digital imaging methods used for measuring [Ca2+]cyt are described in our previous publications (41, 60). Briefly, cells were plated on 25-mm coverslips and were incubated in culture medium containing 3.3 μM fura 2-AM for 30–40 min at room temperature (22–24°C) under an atmosphere of 10% CO2 in air. Fluorescent images were obtained with a microchannel plate image intensifier (Amperex XX1381; Opelco, Washington, DC) coupled by fiber optics to a Pulnix charge-coupled device video camera (Stanford Photonics, Stanford, CA). Image acquisition and analysis were performed with a Metamorph Imaging System (Universal Imaging). [Ca2+]cyt was calculated from fura 2 fluorescence emission excited at 380 and 360 nm by the ratio method (34).

Paracellular tracer flux assay. Flux assays were performed with the 12-mm Transwell as described by Wong and Gumbiner (63) with minor modification. Briefly, IEC-6 or differentiated IEC-Cdx2L1 cells were grown in control cultures or cultures containing DFMO or DFMO plus spermidine for 4 days and then trypsinized, plated at a confluent density of 4 × 105 cells/cm2 on the insert, and maintained under the same culture conditions for an additional 48 h to establish tight monolayers. Two different membrane-impermeable

### Table 1. Oligonucleotide sequences of primers used for RT-PCR

<table>
<thead>
<tr>
<th>Names</th>
<th>Size, bp</th>
<th>Sense/Antisense</th>
<th>Location, nt</th>
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<tr>
<td>E-cadherin</td>
<td>454</td>
<td>5’-GGTTATTCCTGGCATGAGCT-3’</td>
<td>561–580</td>
</tr>
<tr>
<td>(Z 13009)</td>
<td></td>
<td>5’-CTTGCTGGAGTTGCTGTA-3’</td>
<td>998–1016</td>
</tr>
<tr>
<td>β-Catenin</td>
<td>363</td>
<td>5’-TGAAAAATGCTACTGAAGTC-3’</td>
<td>–6–8</td>
</tr>
<tr>
<td>(AF 121265)</td>
<td></td>
<td>5’-GGATTGAGCGCGCTTTCACGATGCT-3’</td>
<td>334–357</td>
</tr>
<tr>
<td>α-Catenin</td>
<td>733</td>
<td>5’-GAGCCCTCTGCGAAAGAA-3’</td>
<td>2655–2672</td>
</tr>
<tr>
<td>(XM 069818)</td>
<td></td>
<td>5’-GCCCATCTGCGACACTGAT-3’</td>
<td>292–309</td>
</tr>
<tr>
<td>ZO-1</td>
<td>249</td>
<td>5’-GCCCTTCGAATAGCATGAT-3’</td>
<td>1571–1590</td>
</tr>
<tr>
<td>(XM 068518)</td>
<td></td>
<td>5’-GATGAAAGCGCCATGATGAT-3’</td>
<td>2009–2031</td>
</tr>
</tbody>
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The GenBank accession numbers (in parentheses) for the sequence were used in designing the primers. ZO, zona occludens.
molecules, [14C]mannitol (mol wt 184) and [3H]inulin (mol wt 5,200), served as paracellular tracers in this experiment. At the beginning of the flux assay, both sides of the bathing wells of Transwell filters were replaced with fresh medium containing either 5 mM unlabeled mannitol or 0.5 mM unlabeled inulin. Each of the tracers was added to a final concentration of 3.6 nM for [14C]mannitol and 0.36 nM for [3H]inulin to the apical bathing wells that contained 0.5 ml of medium. The basal bathing well had no added tracers and contained 1.5 ml of the same flux assay medium as in the apical compartment. All flux assays were performed at 37°C, and the basal medium was collected 2 h after addition of [14C]mannitol or [3H]inulin for counting in a Beckman liquid scintillation counter. The results were expressed as percentage of total count values of each tracer.

**RESULTS**

Effect of polyamine depletion on expression of E-cadherin, β-catenin, α-catenin, ZO-1, and ZO-2 in IEC-6 cells. To determine the involvement of polyamines in regulation of cell-cell adherens junctions and tight junctions, IEC-6 cells were grown in the presence or absence of DFMO, a highly specific inhibitor of polyamine synthesis. Levels of E-cadherin, β-catenin, α-catenin, ZO-1, and ZO-2 mRNAs and proteins were examined 4, 6, and 8 days thereafter. Treatment with 5 mM DFMO completely depleted putrescine within 48 h, but it took 4 days to totally deplete spermidine and significantly deplete spermine (by ~60%). Similar results were published previously (26, 59).

As shown in Fig. 1, depletion of cellular polyamines by DFMO selectively and significantly inhibited mRNA expression of E-cadherin but negligibly affected levels of β-catenin, α-catenin, ZO-1, and ZO-2 mRNAs. The levels of E-cadherin mRNA in the cells treated with DFMO for 4, 6, and 8 days were decreased by ~40% (Fig. 1B). The changes in E-cadherin mRNA were paralleled by those of E-cadherin proteins (Fig. 2). The levels of E-cadherin protein in the cells exposed to DFMO for 4, 6, and 8 days were decreased by ~70% (Fig. 2B, a). Although there was no significant inhibition of β-catenin and α-catenin mRNA expression in DFMO-treated cells, protein levels of these two adherens junction proteins decreased significantly at 6 and 8 days after polyamine depletion (Fig. 2A, B, b, and c). The levels of β-catenin and α-catenin proteins were decreased by ~30% and ~20%, respectively. On the other hand, polyamine depletion had no significant effects on expression of ZO-1, ZO-2, and JAM-1 proteins (Fig. 2A). In the presence of DFMO, decreased levels of E-cadherin mRNA and protein and β-catenin and α-catenin proteins were completely prevented by addition of exogenous spermidine (5 μM). Levels of E-cadherin, β-catenin, and α-catenin expression in cells grown in the presence of DFMO plus spermidine were indistinguishable from those of control cells. We
also examined changes in claudin-1, claudin-4, and occludin proteins in the presence or absence of DFMO for 4, 6, and 8 days and demonstrated that basal levels of these proteins were very low and undetectable by Western blot analysis in IEC-6 cells (data not shown). To verify the sensitivity of the assay system we used in these studies, our methods clearly detected claudin-1, claudin-4, and occludin proteins in positive control samples (data not shown).

**Effects of polyamine deficiency on cellular distribution of E-cadherin, β-catenin, α-catenin, ZO-1, and ZO-2 proteins.** To extend these positive depletion, immunochemical results of E-cadherin, β-catenin, α-catenin, ZO-1, and ZO-2 proteins after polyamine depletion, immunochemical results of these proteins were very low and undetectable by Western blot analysis in IEC-6 cells (data not shown). To verify the sensitivity of the assay system we used in these studies, our methods clearly detected claudin-1, claudin-4, and occludin proteins in positive control samples (data not shown).

![Representative autoradiograms of Western blots.](image)

**A:** As an internal control for equal loading, actin (42 kDa) was identified by probing nitrocellulose with specific antibodies. After the blot was stripped, actin (42 kDa), ZO-2 (160 kDa), and JAM-1 (92 kDa), E-cadherin (102 kDa), β-catenin (92 kDa), ZO-1 (120 kDa), and α-catenin (120 kDa) were identified as expected. Exogenous spermidine given together with DFMO restored [Ca2+]cyt to normal levels. Values are means ± SE of data from 3 separate experiments; relative levels of E-cadherin, β-catenin, and α-catenin proteins were corrected for loading as measured by densitometry of actin. *P < 0.05 vs. control and DFMO + SPD.

**B:** Quantitative analysis derived from densitometric analysis of autoradiograms from cells described in A. A: E-cadherin. B: β-Catenin. C: α-Catenin. Open bars, control; gray bars, DFMO; filled bars, DFMO + SPD. Values are means ± SE of data from 3 separate experiments; relative levels of E-cadherin, β-catenin, and α-catenin proteins were corrected for loading as measured by densitometry of actin. *P < 0.05 vs. control and DFMO + SPD.

We also examined the effect of polyamine depletion on cellular distribution of tight junction proteins in IEC-6 cells. The immunoreactivities for ZO-1 protein were primarily observed along the entire cell-cell contact region of adjacent cells in IEC-6 cells (Fig. 3, bottom). Polyamine depletion did not alter cellular organization of ZO-1 protein. There were no significant differences in cellular distribution and immunostaining levels of ZO-1 protein between control cells and cells treated with DFMO in the presence or absence of spermidine. Similarly, polyamine depletion also did not affect cellular distribution of ZO-2 protein in IEC-6 cells (data not shown).

**Effect of [Ca2+]cyt on E-cadherin expression.** We recently demonstrated that polyamines modulate [Ca2+]cyt by governing the driving force for Ca2+ influx via controlling activity of Kv channels (41, 43, 60) and that Ca2+ mediates distinct actions of intestinal epithelial cells after polyamine increase (15, 40). In this study, we tested the possibility that polyamines regulate E-cadherin expression by altering [Ca2+]cyt. Because IEC-6 cells do not express L-type VDCC (43, 60), the inhibition of Kv channel expression and resultant membrane depolarization through polyamine depletion decrease the driving force for Ca2+ influx and reduce [Ca2+]cyt. In fact, [Ca2+]cyt in cells exposed to DFMO for 6 days decreased significantly (from 115 ± 6.4 to 60.3 ± 3.2 nM; n = 12, P < 0.05). Spermidine given together with DFMO restored [Ca2+]cyt to normal levels.

In the first study, we examined whether the decreased [Ca2+]cyt caused by removal of extracellular...
Ca^{2+} alters expression of E-cadherin mRNA and protein in control cells (without DFMO). As shown in Figs. 4A and 5A, removal of extracellular Ca^{2+} not only decreased E-cadherin mRNA but also inhibited E-cadherin protein expression. This inhibitory effect on E-cadherin expression occurred at 4 h and peaked at 6 h after exposure to the Ca^{2+}-free medium. Levels of E-cadherin mRNA and protein were decreased by ~60% at 4 h and by ~90% at 6 h, respectively, after incubation with the Ca^{2+}-free medium. Some small protein fragmentations were regularly detected in cells exposed to the Ca^{2+}-free medium for 4 and 6 h, probably resulting from the stimulation of E-cadherin degradation. The regulatory effect of Ca^{2+} on E-cadherin is specific because expression of β-catenin protein was not affected after removal of extracellular Ca^{2+} (Fig. 5A, a). There were no significant differences in levels of β-catenin protein between control cells and cells exposed to Ca^{2+}-free medium for 2, 4, and 6 h.

In the second study, we determined whether increased [Ca^{2+}]_{cyt} caused by the Ca^{2+} ionophore ionomycin prevents the inhibition of E-cadherin expression in polyamine-deficient cells. Consistent with our previous findings (15, 41, 43), exposure to 1 μM ionomycin dramatically increased [Ca^{2+}]_{cyt} in DFMO-treated cells (data not shown). Increased [Ca^{2+}]_{cyt} caused by ionomycin also increased levels of E-cadherin mRNA (Fig. 4B) and protein (Fig. 5B) in the absence of polyamines. Treatment of polyamine-deficient cells with ionomycin for 6 h restored E-cadherin mRNA to ~85% of control value and its protein to ~70%. Neither the Ca^{2+}-free medium nor ionomycin affected cell attachment and cell viability in control and DFMO-treated cells as measured by Trypan blue staining (data not shown). These results indicate that polyamines regulate E-cadherin expression, at least partially, through [Ca^{2+}]_{cyt} in intestinal epithelial cells.

**Effects of polyamine depletion and [Ca^{2+}]_{cyt} on E-cadherin protein stability.** To keep this study focused, we specifically examined changes in E-cadherin protein stability in the presence or absence of polyamines. Although E-cadherin is relatively stable, the levels of E-cadherin protein in control cells declined gradually after protein synthesis was inhibited by administration of cycloheximide, with a half-life of ~900 min as calculated by linear regression analysis (Fig. 6, A, a, and B). In DFMO-treated cells, the stability of E-cadherin protein decreased significantly compared with that observed in control cells (Fig. 6A, a vs. b). The half-life of E-cadherin protein in polyamine-deficient cells was ~330 min (Fig. 6B). Spermidine given together with DFMO completely prevented the decreased stability of E-cadherin protein, and levels of E-cadherin in cells exposed to DFMO plus spermidine decreased at a rate similar to that observed in controls.

To validate the methods used in this study and confirm these interesting findings, we also examined the time course for the degradation of E-cadherin in control and polyamine-deficient cells by pulse-chase analysis. As shown in Fig. 7, after the 60-min pulse, E-cadherin protein decreased at a relatively slow rate, with ~65% still remaining after 6 h. This result is similar to that obtained from the cycloheximide method described in Fig. 6. In contrast to that observed...
cellular RNA was harvested, and E-cadherin mRNA levels were assessed and then incubated in Ca\(^{2+}\) medium. Cells were grown in DMEM containing no DFMO for 6 days to examine changes in E-cadherin mRNA expression. 

Fig. 4. Effects of manipulating intracellular Ca\(^{2+}\) concentration, either decreased by removal of extracellular Ca\(^{2+}\) or increased by the Ca\(^{2+}\)-ionophore ionomycin (Iono), on E-cadherin mRNA expression. A: changes in E-cadherin mRNA in control cells exposed to Ca\(^{2+}\)-free medium. Cells were grown in DMEM containing no DFMO for 6 days and then incubated in Ca\(^{2+}\)-free medium for different times. Total cellular RNA was harvested, and E-cadherin mRNA levels were assayed by RT-PCR analysis. a: PCR-amplified products displayed in agarose gels for E-cadherin (454 bp) and β-actin (244 bp). b: Quantitative analysis of RT-PCR results by densitometry from cells described in A. a. Data were normalized to the amount of β-actin (optical density of the E-cadherin mRNA/optical density of the β-actin mRNA) and are expressed as means ± SE from 3 separated experiments. *P < 0.05 vs. 0 h group. B: changes in E-cadherin mRNA in polyamine-deficient cells exposed to ionomycin. Cells were grown in the presence of DFMO for 6 days and then exposed to 1 μM ionomycin for 6 h. a: PCR-amplified products displayed in agarose gels for E-cadherin and β-actin. b: Quantitative analysis of RT-PCR results by densitometry from cells described in B. a. Open bars, control; gray bars, DFMO; filled bars, DFMO + ionomycin. Values are means ± SE of data from 3 separate experiments. *P < 0.05 vs. control group; *P < 0.05 vs. DFMO-treated cells.

Because polyamine depletion decreased [Ca\(^{2+}\)]\(_{\text{cyt}}\), we further determined the involvement of [Ca\(^{2+}\)]\(_{\text{cyt}}\) in E-cadherin protein stability in IEC-6 cells. As shown in Fig. 8, removal of extracellular Ca\(^{2+}\) from the culture medium dramatically decreased E-cadherin stability when protein synthesis was inhibited by cycloheximide, with a half-life of ~80 min. Some small fragmentations of E-cadherin protein were regularly detected in cells exposed to Ca\(^{2+}\)-free medium for 2, 3, 4, and 6 h (Fig. 8A, b). Together, these results indicate that polyamines and [Ca\(^{2+}\)]\(_{\text{cyt}}\) are necessary for maintenance of E-cadherin protein stability in intestinal epithelial cells, suggesting that the reduction of E-cadherin by polyamine depletion results, at least partially, from instability of E-cadherin protein after decrease in [Ca\(^{2+}\)]\(_{\text{cyt}}\).

Effects of decrease in E-cadherin expression by polyamine depletion on paracellular permeability in IEC-6 cells. To study the possible role of decreased E-cadherin in intestinal epithelial paracellular barrier function, we assessed the paracellular flux of membrane-impermeable tracers across the confluent monolayer in the presence or absence of polyamines. Cells were grown in control cultures or cultures containing DFMO or DFMO plus spermidine for 4 days and then plated at confluent density on the insert and maintained for an additional 48 h to establish a tight monolayer. Two widely accepted hydrophilic paracellular tracer molecules, \([^{14}\text{C}]\text{mannitol}\) and \([^{3}\text{H}]\text{inulin}\), were used in this study. To verify the system used for paracellular permeability assays, the effects of decreased [Ca\(^{2+}\)]\(_{\text{cyt}}\) on the paracellular flux of \([^{14}\text{C}]\text{mannitol}\) and \([^{3}\text{H}]\text{inulin}\) were tested and served as a positive control. As expected, exposure to Ca\(^{2+}\)-free medium for 2 h markedly increased paracellular permeability in normal cells (without DFMO) (Fig. 9B, left). Consistent with the effect on E-cadherin expression, polyamine depletion also significantly increased paracellular permeability (Fig. 9B, right). In DFMO-treated cells, levels of paracellular flux of \([^{14}\text{C}]\text{mannitol}\) and \([^{3}\text{H}]\text{inulin}\) were increased by ~30% and ~25%, respectively. Spermidine given together with DFMO restored the paracellular permeability to normal levels. Levels of paracellular permeability in cells exposed to DFMO plus spermidine were similar to those observed in control cells.

Changes in E-cadherin expression and paracellular permeability after polyamine depletion in differentiated IEC-Cdx2L1 cells. To determine whether the inhibition of E-cadherin expression and resultant dysfunction of intestinal epithelial barrier by polyamine depletion occur in differentiated intestinal epithelial cells, we examined changes in levels of E-cadherin protein expression and paracellular permeability in differentiated IEC-Cdx2L1 cells. Our previous studies (42) and others (48) demonstrated that forced expression of the Cdx2 gene in undifferentiated parental IEC-6 cells
induces the development of a differentiated phenotype. After treatment with the gene expression inducer IPTG for 16 days, these stable IEC-Cdx2L1 cells were polarized, showed multiple morphological characteristics of villus-type enterocytes, and highly expressed brush border enzymes such as sucrase-isomaltase. Consistent with parental IEC-6 cells, depletion of cellular polyamines by DFMO also inhibited expression of E-cadherin protein (Fig. 10A) and increased paracellular permeability (Fig. 10C) in differentiated IEC-Cdx2L1 cells. E-cadherin protein in cells exposed to DFMO for 4, 6, and 8 days was decreased by ~45% (Fig. 10B), and levels of paracellular permeability were increased by ~20% in [14C]mannitol and ~80% in [3H]inulin (Fig. 10C). E-cadherin expression and paracellular permeability were returned to normal levels when DFMO was given together with spermidine. These results indicate that polyamines are essential for the E-cadherin expression that is absolutely required for integrity of intestinal epithelial paracellular barrier function.

**DISCUSSION**

Polyamines are essential for maintenance of normal intestinal mucosal integrity, but few specific functions of polyamines in this process have been defined. In the present study, parental IEC-6 cells and differentiated IEC-Cdx2L1 cells were used to determine the role of polyamines in the expression of intercellular junction proteins and the regulation of epithelial barrier function. The results of these studies indicate that depletion of cellular polyamines by DFMO inhibits expression of the adherens junction proteins E-cadherin, β-catenin, and α-catenin but does not alter levels and cellular distribution of the tight junction proteins ZO-1, ZO-2 and JAM-1. Decreased expression of these adherens junction proteins was associated with a significant increase in epithelial paracellular permeability in both IEC-6 and differentiated IEC-Cdx2L1 cells, suggesting dysfunction of intestinal epithelial barrier after polyamine depletion. These results provide, for the first time, new evidence demonstrating that polyamines regulate intestinal epithelial barrier by altering expression of adherens junction proteins.

It has been recognized that cell-cell adhesion, cytoskeleton, and signaling transduction functions overlap at the adherens junction to integrate a variety of cellular information and regulate intestinal epithelial barrier function. The cytoplasmic domain of E-cadherin directly associates with β-catenin in intestinal epithelial cells, and this cadherin-catenin complex is linked through α-catenin either directly or indirectly to the actin cytoskeleton (4, 50). This association of the cad-

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**Fig. 5.** Changes in E-cadherin protein expression in cells described in Fig. 4. A: levels of E-cadherin protein in control cells exposed to Ca^{2+}-free medium. Whole cell lysates were harvested at various times after exposure to Ca^{2+}-free medium, and E-cadherin levels were assayed by Western blot analysis. *: Representative autoradiograms of Western blots for E-cadherin (~120 kDa) and β-catenin (~92 kDa). After the blot was stripped, actin (~42 kDa) immunoblotting was performed as an internal control for equal loading. a: Representative autoradiograms of Western blots for E-cadherin (~120 kDa) and β-catenin (~92 kDa). After the blot was stripped, actin (~42 kDa) immunoblotting was performed as an internal control for equal loading. b: Quantitative analysis of Western immunoblots by densitometry from cells described in A, a. Open bar, no exposure to Ca^{2+}-free medium; filled bars, exposure to Ca^{2+}-free medium. Values are means ± SE of data from 3 separate experiments; relative E-cadherin protein levels were corrected for loading as measured by densitometry of actin. *P < 0.05 vs. 0 h group. B: levels of E-cadherin protein in polyamine-deficient cells exposed to ionomycin for 6 h. a: Representative autoradiograms of Western blots. b: Quantitative analysis of Western immunoblots by densitometry from cells described in B, a. Open bars, control; gray bars, DFMO; filled bars, DFMO + ionomycin. Values are means ± SE of data from 3 separate experiments. *P < 0.05 vs. control group; *P < 0.05 vs. DFMO-treated cells.
herin-catenin complex with the actin filament network is crucial for strong cell-cell adhesions and has a functional role in forming the intestinal epithelial barrier. During the assembly of different intercellular junctions, cadherin-catenin complexes are stabilized at sites of cell-cell contact and subsequently develop into mature adherens junctions (1, 3, 19, 46). Inhibition of adherens junctions by either treatment with the specific antibody against E-cadherin (4, 29) or ectopic expression of a dominant-negative mutant E-cadherin (54, 61) retards the assembly of tight junctions and disrupts epithelial paracellular barrier functions. 

In contrast, stimulation of E-cadherin expression by exposure of gastric epithelium to a low-pH environment enhances adherens junction formation and stabilizes the epithelial barrier against acid back-diffusion (32). Consistent with observations in epithelial cells, overexpression of a mutated nonfunctional N-cadherin inhibits endothelial adhesions, impairs tight junction assembly, and decreases vascular endothelial barrier function (2, 11). More recently, it was found that overexpression of a mutant VE-cadherin lacking an adhesive extracellular domain disrupts endothelial barrier function and also inhibits endothelial growth in dermal microvascular endothelial cells (54).

The findings reported here indicate that expression of adherens junction proteins in normal intestinal epithelial cells requires polyamines and that polyamine depletion by DFMO dramatically decreases levels of both E-cadherin mRNA and protein and, to a lesser degree, also inhibits expression of β-catenin and...
α-catenin proteins, suggesting that polyamines have distinct roles in expression of different adherens junction proteins. This result is not surprising, because polyamines are implicated in multiple signaling pathways and involved at different levels in the expression of various genes in intestinal epithelial cells. For example, polyamines stimulate the transcription of c-myc and c-jun but have no effect on these two genes posttranscriptionally (36). On the other hand, polyamines destabilize the mRNAs of transforming growth factor (TGF)-β (35), p53 (26), and junD (24) genes without affecting their transcriptional rates. In addition, the regulatory effects of polyamines on expression of adhe-

![Image](https://example.com/image1)

Fig. 8. Effect of exposure to Ca²⁺-free medium on E-cadherin protein stability in normal IEC-6 cells. After cells were grown in DMEM containing no DFMO for 6 days, they were exposed to Ca²⁺-free medium and cycloheximide at a concentration of 50 μg/ml at the same time, and whole cell lysates were harvested at indicated periods. E-cadherin protein levels were assayed by Western blot analysis, and loading of proteins was monitored by actin. A: representative autoradiograms of Western blots of control cells (a) and cells exposed to Ca²⁺-free medium (b). B: quantitative analysis of Western immunoblots by densitometry from cells described in A. Values are means ± SE of data from 3 separate experiments, and relative levels of E-cadherin were corrected for protein loading as measured by densitometry of actin.

![Image](https://example.com/image2)

Fig. 9. Changes in paracellular permeability in IEC-6 cells described in Fig. 6. A: diagram depicting measurement of paracellular permeability. Cells were cultured on the filter of the insert, and membrane-impermeable tracer molecules, ¹⁴C-labeled mannitol (mol wt 184) and ³H-labeled inulin (mol wt 5,200), were added to the insert medium. Paracellular tracer flux was measured in the basal medium. B: paracellular permeability in normal cells exposed to Ca²⁺-free medium (left) or cells treated with DFMO or DFMO + SPD (right). a: [¹⁴C]mannitol permeability. b: [³H]inulin permeability. After cells were grown in control cultures or cultures containing either 5 mM DFMO alone or DFMO + 5 μM SPD for 4 days, they were trypsinized, plated at confluent density on the insert, and then maintained under the same culture conditions for additional 48 h. The entire basal medium was collected 2 h after addition of [¹⁴C]mannitol or [³H]inulin for paracellular tracer flux assays. Values are means ± SE of data from 8 samples. *P < 0.05 vs. control group; †P < 0.05 vs. DFMO-treated cells.
It is interesting and of biological significance that polyamine depletion decreases expression of adherens junction proteins, particularly E-cadherin, leading to dysfunction of the intestinal epithelial barrier. On one hand, intestinal epithelial cells contain high levels of polyamines, and the intracellular polyamine pool is critically controlled by polyamine synthesis, uptake, and degradation (49, 56, 57). Cellular polyamines increase rapidly in response to various physiological and pathogenic stimuli and affect distinct epithelial functions. On the other hand, adherens junctions integrate various intracellular and extracellular signals in regulation of the epithelial barrier and are vulnerable under various pathological conditions. It has been shown that E-cadherin is the target for cadmium toxicity in intestinal epithelial cells and that exposure to cadmium chloride damages E-cadherin-mediated cell-cell adhesions and decreases epithelial barrier function (10, 38). Expression of adherens junctions in patients with inflammatory bowel disease (IBD) is generally more affected by active inflammation than that of tight junctions and desmosomes (12, 18, 20, 22). Levels of E-cadherin and α-catenin in actively inflamed IBD tissues are dramatically reduced in epithelial cells, whereas several tight junction- and desmosome-associated proteins remain to express at normal levels. This decreased expression of adherens junctions is associated with a significant change in intestinal epithelial paracellular permeability in these patients (12, 22).

The data from the current studies also show that polyamines regulate expression of the E-cadherin gene, at least partially, through Ca\(^{2+}\). Polyamine depletion by DFMO reduced [Ca\(^{2+}\)]\(_{cyt}\) and decreased levels of E-cadherin mRNA and protein. As noted in Fig. 4, elevation of [Ca\(^{2+}\)]\(_{cyt}\), by the Ca\(^{2+}\) ionophore ionomycin restored E-cadherin mRNA to near normal in polyamine-deficient cells, whereas reduction of [Ca\(^{2+}\)]\(_{cyt}\) through the removal of extracellular Ca\(^{2+}\) decreased the level of E-cadherin mRNA in normal cells (without DFMO), suggesting that polyamine-regulated [Ca\(^{2+}\)]\(_{cyt}\) is implicated in the regulation of either E-cadherin mRNA synthesis or mRNA degradation or both. The current studies further show that polyamine-regulated [Ca\(^{2+}\)]\(_{cyt}\) is also implicated in the posttranslational regulation of E-cadherin. Reduction of [Ca\(^{2+}\)]\(_{cyt}\) by polyamine depletion or exposure to Ca\(^{2+}\)-free medium significantly decreased the stability of E-cadherin protein, as indicated by a decrease in its protein half-life (Figs. 6–8). This finding that polyamines are involved in the maintenance of E-cadherin protein stability via Ca\(^{2+}\) is consistent with our previous studies (41) that demonstrate that stabilization of the RhoA protein by polyamines is mediated by Ca\(^{2+}\) in intestinal epithelial cells.

The results presented in Figs. 9 and 10 indicate that decreased expression of adherens junction after polyamine depletion increases intestinal epithelial paracellular permeability. Our results are consistent with those of others who have demonstrated that disruption of adherens junctions impairs epithelial barrier func-

![Fig. 10. Changes in E-cadherin protein expression and paracellular permeability in differentiated IEC-Cdx2L1 cells grown in control cultures, cultures in which ornithine decarboxylase activity was inhibited with 5 mM DFMO, and cultures inhibited with DFMO and supplemented with exogenous 5 μM SPD. Before experiments, stable transfected-IEC-Cdx2L1 cells were cultured in DMEM containing 4 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 16 days to induce differentiation. These differentiated cells were then grown in DMEM containing 5% dialyzed FBS and 5 mM DFMO with or without 5 μM SPD for 4, 6, and 8 days. A: representative autoradiograms of Western blots for E-cadherin. Whole cell lysates were harvested, and E-cadherin protein levels were assayed with the specific antibody. After the blot was stripped, actin immunoblotting was performed as an internal control for equal loading. B: quantitative analysis derived from densitometric analysis of autoradiograms from cells described in A. Open bars, control; gray bars, DFMO; filled bars, DFMO + SPD. Values are means ± SE of data from 3 separate experiments. *P < 0.05 vs. control groups. C: paracellular permeability in cells exposed to DFMO or DFMO + SPD for 6 days. Paracellular permeability was measured by the methods described in Fig. 9. a: [\(^{14}\)C]mannitol permeability. b: [\(^{3}H\)]inulin permeability. Open bars, control; gray bars, DFMO; filled bars, DFMO + SPD. Values are means ± SE of data from 8 samples. *P < 0.05 vs. control group; #P < 0.05 vs. DFMO-treated cells.](http://ajpcell.physiology.org/)
tion (12, 29, 51, 54). However, most of the studies that used an in vitro system that mimicked the intestinal epithelial barrier used T84 and Caco-2 cells (27, 33, 66). Although these cells are associated with the differentiated phenotype and have well-characterized tight junctions, they are cells of carcinoma origin. In this study, we used IEC-6 cells as a model, which are derived from normal intestinal epithelial cells and highly express adherens junction proteins E-cadherin, β-catenin, and α-catenin as well as tight junction proteins ZO-1 and ZO-2. Because expression of tight junction proteins ZO-1 and ZO-2 was not affected in DFMO-treated cells, increased paracellular permeability after polyamine depletion results primarily from inhibition of expression of adherens junction proteins. Considering the fact that IEC-6 cells are undifferentiated epithelial cells and lack some tight junction proteins such as occludin (data not shown), differentiated IEC-Cdx2L1 cells were also used in the present experiments. Consistent with the observation in parental IEC-6 cells, polyamine depletion by DFMO also decreased E-cadherin expression and increased epithelial paracellular permeability in differentiated intestinal epithelial cells.

The mechanism by which intestinal epithelial paracellular permeability is increased after inhibition of adherens junction protein expression in polyamine-deficient cells is unknown. Because tight junctions seal epithelial cells together and prevent leaking between cells, it is possible that disruption of adherens junctions by polyamine depletion inhibits tight junction assembly in intestinal epithelial cells. Numerous intracellular signals have been shown to regulate tight junction assembly and function, and some of these signaling transduction proteins are associated with the adherens junctions (4, 65). Two models are proposed to delineate how adherens junctions regulate tight junction assembly, although they are not mutually exclusive. The first model hypothesizes that the cadherin-catenin complex in the adherens junctions physically nucleates tight junction assembly. In support of this hypothesis, inhibition of cadherin adhesions prevents tight junction assembly (13, 61). The second model suggests that adherens junctions activate specific intracellular signaling pathways such as protein kinase C and Rho-family GTPases, which then activate tight junction assembly (4, 7). Impairment of adherens junctions could alter the balance of these signaling systems, thus leading to disruption of tight junction function. In addition, a third possibility also exists, suggesting that the effects of polyamines and Ca2+ could act independently on the adherens junctions and tight junctions in intestinal epithelial cells. It is not clear at present whether tight junction assembly is inhibited after decreased expression of adherens junctions in polyamine-deficient cells and whether other signaling transduction proteins are also involved in this process. Clearly, further studies are necessary to determine the exact roles of polyamines and polyamine-mediated expression of adherens junctions in the regulation of tight junction assembly and intestinal epithelial paracellular barrier function.

In summary, these results indicate that polyamines are required for expression of adherens junction proteins and involved in the regulation of intestinal epithelial barrier function. Depletion of cellular polyamines by DFMO decreases expression of the adherens junction proteins E-cadherin, β-catenin, and α-catenin but has no effect on the tight junction proteins ZO-1, ZO-2, and JAM-1. This decreased expression of adherens junctions is associated with a significant increase in intestinal epithelial paracellular permeability. These results also show that polyamine depletion reduces [Ca2+]cyt in intestinal epithelial cells and that decreased [Ca2+]cyt caused by either polyamine depletion or exposure to Ca2+-free medium significantly inhibits expression of E-cadherin mRNA and decreases the half-life of E-cadherin proteins. It is indicated that decreased levels of E-cadherin in polyamine-deficient cells result from both decrease in mRNA expression and increase in the rate of its protein degradation. These findings suggest that polyamines regulate intestinal epithelial barrier function by altering expression of adherens junctions, at least partially through [Ca2+]cyt.

DISCLOSURES

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REFERENCES


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Cpe<sup>flt</sup>/Cpe<sup>flt</sup> mice are defective in carboxypeptidase E and pro-insulin processing. *Endocrinology* 138: 4883–4892, 1997.


