Akt Kinase Activation Blocks Apoptosis in Intestinal Epithelial Cells by Inhibiting Caspase-3 after Polyamine Depletion*

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Huifang M. Zhang‡§, Jaladanki N. Rao‡§, Xin Guo‡§, Lan Liu‡§, Tongtong Zou‡§, Douglas J. Turner‡§, and Jian-Ying Wang‡§¶

From the Departments of ‡Surgery and ¶Pathology, University of Maryland School of Medicine and §Baltimore Veterans Affairs Medical Center, Baltimore, Maryland 21201

Apoptosis plays a critical role in the maintenance of gut mucosal homeostasis and is regulated by numerous factors including polyamines. Although the exact roles of polyamines in apoptotic pathway are still unclear, inhibition of polyamine synthesis promotes the resistance of intestinal epithelial cells to apoptosis. Akt is a serine-threonine kinase that has been established as an important intracellular signaling in regulating cell survival. The current studies test the hypothesis that polyamines are involved in the control of Akt activity in normal intestinal epithelial cells (IEC-6 line) and that activated Akt mediates suppression of apoptosis following polyamine depletion. Depletion of cellular polyamines by α-difluoromethylornithine induced levels of phosphorylated Akt and increased Akt kinase activity, although it had no effect on expression of total Akt, pERK, p88, and Bcl-2 proteins. This activated Akt was associated with both decreased levels of active caspase-3 and increased resistance to tumor necrosis factor-α/cycloheximide-induced apoptosis. Inactivation of Akt by either treatment with LY294002 or ectopic expression of a dominant negative Akt mutant (DNAMakt) not only enhanced the caspase-3 activation in polyamine-deficient cells but also prevented the increased resistance to tumor necrosis factor-α/cycloheximide-induced apoptosis. Phosphorylation of glycogen synthase kinase-3, a downstream target of Akt, was also increased in α-difluoromethylornithine-treated cells, which was prevented by inactivation of Akt by LY294002 or DNAMakt overexpression. These results indicate that polyamine depletion induces the Akt activation mediating suppression of apoptosis via inhibition of caspase-3 in normal intestinal epithelial cells.

The epithelium of the intestinal mucosa has the most rapid turnover rate of any tissue in the body and its integrity depends on a dynamic balance between cell proliferation, growth arrest, and apoptosis (1–4). Undifferentiated intestinal epithelial cells continuously replicate in the proliferative zone within the crypts and differentiate as they migrate up the luminal surface of intestine to replace lost cells under physiological conditions (5, 6). It has been shown that apoptosis, rather than simple exfoliation of enterocytes, accounts for the majority of cell loss at the luminal surface of the colon and villous tips in the small intestine (4, 7). Apoptosis also occurs in the crypt into which this process maintains the balance in cell number between newly divided and surviving cells (3, 4, 7). To maintain tissue homeostasis of the mucosa, the rates of epithelial cell division and apoptosis must be highly regulated. Increasing evidence indicates that the natural polyamines spermidine and spermine and their precursor, putrescine, are the central convergence point for the multiple signaling pathways driving different epithelial cell functions. We (8–10) and others (11, 12) have shown that normal intestinal epithelial growth depends on the supply of polyamines to the dividing cells in the crypts, and that differentiated epithelial cells of the mucosal surface are also exposed to the high concentrations of polyamines in the luminal contents of the intestine (13). Recently, polyamines have been implicated in the control of the apoptotic response in intestinal epithelial cells (14–16). However, the exact mechanisms by which polyamines are involved in the regulation of apoptosis are still unclear.

The serine-threonine kinase Akt, also known as protein kinase B (PKB),1 is the cellular homologue of the retroviral oncogene v-Akt and has been established as a multifunctional signaling intermediate in the regulation of apoptosis, cell cycle progression, and energy metabolism (17, 18). To date, there are three mammalian isoforms of Akt proteins: Akt1 (PKBα), Akt2 (PKBβ), and Akt3 (PKBγ), which display more than 80% sequence homology (19). All three isoforms of Akt contain a pleckstrin homology domain at the N terminus, followed by a catalytic domain related to PKA and PKC, and a regulatory region at the C terminus. The catalytic domain of Akt contains the first phosphorylation site, Thr308, and the second phosphorylation site, Ser473, located in the C-terminal tail (20). Phosphorylation of both Thr308 and Ser473 is required for full enzyme activity of Akt (18, 21). Recently, an exciting insight into the function of Akt is its involvement in the regulation of apoptosis. Akt has been shown to positively mediate cell survival in skeletal muscle (22), neurons (23), endothelial (24, 25), and epithelial cells (26, 27). Akt activation is induced by several growth factors including nerve growth factor, platelet-derived growth factor, basic fibroblast growth factor, insulin and insulin-like growth factor-1, which results in receptor tyrosine ki-

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1 The abbreviations used are: PKB, protein kinase B; DFMO, difluoromethylornithine; TNF-α, tumor necrosis factor-α; CHX, cycloheximide; IEC, intestinal epithelial cells; FBS, fetal bovine serum; ERK, extracellular signal-regulated kinase; DMEM, Dulbecco’s modified Eagle’s medium; PARP, poly(ADP-ribose) polymerase; DFI, DNA fragmentation factor inhibitor; GSK-3, glycogen synthase kinase-3; MOPS, 4-nor-ephedrinepropanesulfonic acid; PI3K, phosphatidylinositol 3-kinase.

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Akt phosphorylation (21). Activated Akt phosphorylates several downstream targets, including Bad, caspase-9, and forkhead transcription factor FKHR, thus altering their pro-apoptotic or anti-apoptotic functions (28–30). In addition, many other members of the apoptotic machinery as well as transcription factors contain the Akt consensus phosphorylation site (17, 18), further suggesting that Akt play a crucial role in cell survival.

Although the exact roles of polyamines in apoptotic pathways have been rather controversial, depending on the cell type and death stimulus, we (10, 31) have recently demonstrated that polyamine depletion by inhibition of ornithine decarboxylase, the first rate-limiting step in polyamine synthesis, with difluoromethylornithine (DFMO) promotes the resistance to TNF-α/cycloheximide (CHX)-induced apoptosis in normal intestinal epithelial cells (IEC-6 line). Given the distinct roles of cellular polyamines in regulation of intestinal epithelial functions and that Akt signaling is a key control point of cell survival, the current studies were to test the hypothesis that polyamines are involved in the control of Akt activity in intestinal epithelial cells and the activated Akt mediates the increased resistance to TNF-α/CHX-induced apoptosis following polyamine depletion. First, we examined whether polyamine depletion induced Akt phosphorylation and increased its kinase activity in IEC-6 cells. Second, we determined whether inactivation of Akt by either treatment with the specific chemical inhibitor LY294002 or ectopic expression of the dominant negative Akt mutant prevented the increased resistance of polyamine-deficient cells to TNF-α/CHX-induced apoptosis. Third, we tested the possibility that activated Akt induced intestinal epithelial cell survival by inhibiting caspase-3 activity following polyamine depletion. Some of these data have been published previously in abstract form (32).

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 (FBS) were obtained from Invitrogen, and biochemicals were obtained from Sigma. Antibodies against phosphorylated Akt (p-Akt at serine 473), total Akt, phosphorylated extracellular signal-regulated kinase (pERK), and phosphorylated-p38 (p-p38) at Thr180/Tyr182/183 were purchased from Cell Signaling (Beverly, MA). Secondary antibodies conjugated to horseradish peroxidase were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). DL-difluoromethylornithine (DFMO) was purchased from Ilex Oncology Inc. (San Antonio, TX). LY294002 and the non-radioactive Akt kinase assay kit were purchased from Cell Signaling.

Plasmid Construction and Transfection—The full-length cDNA of the dominant negative Akt mutant (DNMAkt), containing a Myc-His tag at its 3’ end and a substitution of methionine (ATG) for lysine (AAG) at residue 179 (33), was inserted into the Klenow-blunted Nhel and Pmel sites of expression vector pUSEEamp(+). The plasmid was purchased from the University of California, Santa Barbara (34). Stock cells were transfected with the transfection medium (DMEM supplemented with 5% heat-inactivated FBS, 10 μg of insulin/ml, and 50 μg/ml of gentamicin. Flasks were incubated at 37°C in a humidified atmosphere of 90% air, 10% CO2, and cultures passages 15–20 were used in experiments. There were no significant changes of biological function and characterization of IEC-6 cells at passages 15–20 (data not shown).

In the first series of studies, we examined whether polyamine depletion increased Akt phosphorylation and its kinase activity in IEC-6 cells. The general protocol of the experiments and methods were similar to those described previously (10, 31). Briefly, IEC-6 cells were plated at 6.25 × 104 cells/cm2 and grown in control medium (DMEM + 5% dialyzed FBS + 10 μg of insulin and 50 μg of gentamicin/mL) or the DMEM containing 5 mM DFMO or DFMO plus 10 μM potas pine for 2, 4, and 6 days. The dishes were placed on ice, the monolayers were washed three times with ice-cold Dulbecco’s phosphate-buffered saline, and then different solutions were added according to the assays to be conducted. Levels of p-Akt, total Akt, pERK, p-p38, and Bcl-2 proteins were measured by Western blot analysis and activity of the Akt kinase was examined by using non-radioactive Akt kinase assay kit.

In the second series of studies, we investigated whether induced Akt activity played a role in the increased resistance of polyamine-deficient cells to TNF-α/CHX-induced apoptosis. Akt activation in polyamine-deficient cells was specifically prevented by either pretreatment with the chemical inhibitor LY294002 or stable DNMAkt transfection. IEC-6 cells were grown in the presence of DFMO for 6 days and LY294002 at a concentration of 20 μM was added to the medium during the last 24 h. Apoptosis was induced by exposure to TNF-α in combination with CHX. In studies dealing with DNMAkt, stable DNMAkt-transfected IEC cells were treated with 5 mM DFMO for 6 days and then exposed to TNF-α/CHX. Apoptotic cell death was examined 4 h after treatment with TNF-α/CHX.

In the third series of studies, we examined the involvement of caspase-3 activity in the mechanism by which activated Akt mediates suppression of TNF-α/CHX-induced apoptosis in polyamine-deficient cells. IEC-6 cells were initially grown in cultures containing 5 mM DFMO for 5 days, treated with 20 μM LY294002 for 24 h in the presence of DFMO, and then exposed to TNF-α/CHX. Stable DNMAkt-transfected IEC cells were grown in the presence of 5 mM DFMO for 6 days and then treated with TNF-α/CHX for 4 h. Levels of the active form caspase-3, its downstream proteins poly(ADP-ribose) polymerase (PARP), and DFFI were measured 4 h after exposure to TNF-α/CHX.

Western Blot Analysis—Cell samples, dissolved in ice-cold MOPS buffer (25 mM Tris-HCl, pH 8.0, 137 mM NaCl, 1 mM EDTA, 205 mM sodium pyrophosphate, 1 mM NaVO3, 10% glycerol, 1% Triton X-100, 10 mM MgCl2, and 100 μM aprotinin), were sonicated and centrifuged at 14,000 rpm for 15 min at 4°C. The protein concentration of the supernatant was measured by the methods described by Bradford (37), and each lane was loaded with 20 μg of protein equivalent. The supernatant was boiled for 5 min and then subjected to electrophoresis on 10% acrylamide gels according to Laemmli (38). Immunologic evaluation was then performed overnight at 4°C in 5% nonfat dry milk/Tris-buffered saline-Tween 20 buffer containing specific antibodies against p-Akt, total Akt, pERK, p-p38, Bcl-2, caspase-3, PARP, DFFI, pGSK-3, pFKHR, and pBad proteins. The filters were subsequently washed with 1× Tris-buffered saline-Tween 20 and incubated with the secondary antibodies conjugated with horseradish peroxidase for 1 h at room temperature. The immunocomplexes on the filters were reacted for 1 min with chemiluminescence reagent (NEL-100 PerkinElmer Life Sciences).

Measurement of Akt Kinase Activity—Akt kinase activity was examined by using the non-radioactive Akt kinase kit (Cell Signaling) and performed according to the manufacturer’s instructions. Briefly, whole cell lysates from control and polyamine-deficient cells were collected and incubated with anti-Akt antibody at 4°C for 3 h for immunoprecipitation. The pellets were washed twice with 500 μl of lysis buffer and twice with 500 μl of kinase buffer. Exogenous glutathione-S-transferase (GST)-Akt (GST-3) was added to the samples and served as the substrate of p-Akt in this assay. The kinase reaction was carried in a 40-μl kinase buffer containing 200 μM ATP and 1 μg of GST-3 fusion protein at 30°C for 30 min and the reaction was stopped by adding 20 μl of SDS buffer. Akt kinase activity was determined by Western blot analysis using anti-phosphoAkt antibody or anti-Akt antibody.

Assessment of Morphology and Annexin V Staining—After various experimental treatments, cells were photographed with a Nikon inverted microscope before fixation. Annexin V staining of apoptosis was carried out using a commercial apoptosis kit (Clontech Laboratories, Inc., Palo Alto, CA) and performed according to the protocol recommended by the manufacturer. Cells were washed twice with binding buffer, and resuspended in 200 μl of 1× binding buffer. Five μl of annexin V was added on the slide and incubated at room temperature for 10 min in the dark. Annexin-stained cells were visualized and photographed under a fluorescence microscope using a dual filter set for
fluorescein isothiocyanate and rhodamine, and the percentage of "apoptotic" cells was determined.

High Performance Liquid Chromatography Analysis of Cellular Polyamines—The cellular polyamine content was determined as previously described (10, 36). The results are expressed as nanomoles of polyamines per milligram of protein.

Statistics—Values are mean ± S.E. from three to six samples. Autoradiographic results were repeated three times. The significance of the difference between means was determined by analysis of variance. The level of significance was determined using Duncan’s multiple-range test (39).

RESULTS

Effect of Polyamine Depletion on Akt Phosphorylation and Its Kinase Activity in IEC-6 Cells—Our previous studies (31, 40) demonstrated that polyamines regulate susceptibility of intestinal epithelial cells to apoptosis and that depletion of cellular polyamines promotes resistance to TNF-α/CHX-induced apoptosis. The focus of the current study went further to determine the involvement of the Akt signaling pathway in this process. Consistent with our previous studies (10, 36), inhibition of ornithine decarboxylase activity by exposure to 5 mM DFMO almost completely depleted cellular polyamines in IEC-6 cells. Putrescine was undetectable on day 2 after treatment with DFMO. Spermine was significantly decreased on day 2 and was undetectable on day 4. Spermine was less sensitive to DFMO but decreased by ∼65% on days 4 and 6 in the DFMO-treated cells (data not shown). Results presented in Fig. 1 show that polyamine depletion by DFMO increased levels of Akt phosphorylation, although it had no effect on expression of total Akt, p-ERK, pp38, and Bcl-2 proteins. The induction of phosphorylated Akt protein (p-Akt) occurred at day 2 and continuously increased at 4 and 6 days after treatment with DFMO. The levels of p-Akt in DFMO-treated cells were ∼2.3, ∼6.2, and ∼8.6 times the normal values (without DFMO) at 2, 4, and 6 days, respectively (Fig. 1B). Putrescine (10 μM) given together with DFMO inhibited the increase in the levels of p-Akt protein. Addition of spermidine (5 μM) had an effect equal to putrescine on p-Akt when it was added to cultures containing DFMO (data not shown). In addition, although the levels of p-ERK gradually increased with times during cell growth, there were no significant differences between controls and cells treated with DFMO alone or DFMO plus putrescine.

Increased levels of p-Akt in cells exposed to DFMO were paralleled by a significant increase in Akt kinase activity as indicated by increased phosphorylation of exogenous GSK-3 in an in vitro assay (Fig. 2A). The levels of Akt kinase activity were increased by ∼10-fold after 6 days of DFMO treatment, and this effect was completely prevented by putrescine given together with DFMO (Fig. 2B). These results clearly indicate that depletion of cellular polyamines increases Akt phosphorylation and activates its kinase activity in intestinal epithelial cells.

Effect of Inhibition of Phosphatidylinositol 3-Kinase (PI3K) by Treatment with LY294002 on Akt Phosphorylation in Polyamine-deficient Cells—To determine the involvement of PI3K signaling in Akt activation following polyamine depletion, a specific PI3K inhibitor, LY294002 (41), was used in this study. As shown in Fig. 3A, exposure of polyamine-deficient cells to LY294002 dose-dependently inhibited the phosphorylation of Akt protein. When LY294002 at different concentrations was added to the medium for 24 h, levels of p-Akt protein in DFMO-treated cells were decreased by ∼59% at 10 μM, ∼81% at 20 μM, and ∼89% at 50 μM, respectively. This inhibitory effect of LY294002 on Akt phosphorylation in DFMO-treated cells is specific because levels of total Akt protein were not affected (Fig. 3A, a, middle). Results presented in Fig. 3B further show that treatment with LY294002 at concentrations ranging from 10 to 20 μM did not induce a significant formation of the active form caspase-3, although this compound at the concentration of 50 μM slightly induced the cleavage of procaspase-3 to caspase-3 in polyamine-deficient cells. In addition, there was no apparent loss of cell viability in DFMO-treated cells exposed to LY294002 at the concentration of 20 μM for 24 h (data not shown). These findings suggest that polyamine depletion induces activation of Akt through the PI3K-dependent pathway in intestinal epithelial cells.

Effect of LY294002 on the Increased Resistance to TNF-α/CHX-induced Apoptosis following Polyamine Depletion—To elucidate the function of increased Akt activity in polyamine-
deficient cells, the TNF-α/CHX-induced apoptosis model was used. TNF-α is a proinflammatory cytokine that has a potent cytotoxic effect on intestinal epithelial cells and is widely used as an apoptotic inducer (30, 31, 41, 52–54). However, the cytotoxic effects of TNF-α on most cells, including intestinal epithelial cells, are only evident if protein synthesis is inhibited (30, 31, 40, 54), suggesting that de novo protein synthesis protects cells from TNF-α cytotoxicity. Because increased release of TNF-α in the gut mucosa occurs commonly together with down-regulation of protein synthesis under various physiological and pathological conditions (1, 3–7), this model is more applicable to the in vivo condition than most of the apoptosis models employed and is suitable for the current study.

Inhibition of Akt activation by treatment with LY294002 completely blocked the protective effect of polyamine depletion against TNF-α/CHX-induced apoptosis in IEC-6 cells (Fig. 4). When TNF-α (20 ng/ml) together with CHX (25 μg/ml) was added to control cultures, it induced typical apoptotic cell death (Fig. 4A, a versus b, and B, left). Annexin V staining showed significant phosphatidylserine presence in the cell membrane, a classic indicator of apoptotic cells (Fig. 4A, b, right). In DFMO-treated cells, treatment with the same doses of TNF-α/CHX caused no apoptosis. There were no differences in morphological features and percentage of apoptotic cells between cells treated with DFMO alone and DFMO-treated cells exposed to TNF-α/CHX (Fig. 4A, c versus d, and M, middle). However, when polyamine-deficient cells were pretreated with LY294002 at the concentration of 20 μm, the resistance to TNF-α/CHX-induced apoptosis was abolished (Fig. 4A, e versus f, and B, right). Morphological features and annexin V staining in DFMO-treated cells exposed to LY294002 were indistinguishable from those in cells grown in the control cultures after exposure to TNF-α/CHX (Fig. 4A, b versus f).

Changes in Apoptotic Response in Stable DNMAkt-transfected IEC Cells in the Presence or Absence of Cellular Polyamines—To further define the role of induced Akt in the increased resistance of polyamine-deficient cells to TNF-α/CHX-induced apoptosis, stable DNMAkt-transfected IEC cells were developed and characterized. The expression vector encoding the DNMAkt cDNA with a Myc tag under the control of the cytomegalovirus promoter was constructed (Fig. 5A). Inhibition of Akt activation by ectopic expression of DNMAkt increased resistance of polyamine-deficient cells to TNF-α/CHX-induced apoptosis (Fig. 5B), this model is more applicable to the in vivo condition than most of the apoptosis models employed and is suitable for the current study. The expression vector encoding the DNMAkt cDNA with a Myc tag under the control of the cytomegalovirus promoter was constructed (Fig. 5A). Inhibition of Akt activation by ectopic expression of DNMAkt increased resistance of polyamine-deficient cells to TNF-α/CHX-induced apoptosis (Fig. 5B), this model is more applicable to the in vivo condition than most of the apoptosis models employed and is suitable for the current study.
C3, and ~83% in C4 after exposure to TNF-α/CHX (Fig. 6B). In contrast, the percentages of TNF-α/CHX-induced apoptosis in stable wild-type Akt-transfected IEC cells were lower than those of control cells (data not shown). Inactivation of Akt signaling by DNMAkt transfection also prevented the increased resistance to TNF-α/CHX-induced apoptosis following polyamine depletion. As shown in Fig. 7, exposure of control cells that were transfected with the vector containing no DNMAkt cDNA to TNF-α/CHX induced typical apoptotic cell death as that observed in parental IEC-6 cells. Polyamine depletion by DFMO protected the cells against TNF-α/CHX-induced apoptosis as indicated by morphological features and annexin V staining (Fig. 6, A, c versus d, and B). On the other hand, treatment of stable DNMAkt-transfected cells (C3) with DFMO for 6 days did not induce the resistance to apoptosis after exposure to TNF-α/CHX. Morphological features and annexin V staining in DFMO-treated stable DNMAkt-transfected cells were identical with those observed in control cells exposed to TNF-α/CHX (Fig. 7 A, b versus f). The percentage of apoptotic cells was ~61% in DFMO-treated DNMAkt-transfected cells (C3) compared with ~2% in DFMO-treated cells transfected with control vector after exposure to TNF-α/CHX (Fig. 7B). We also examined changes in apoptosis response of other DNMAkt-transfected cell clones (C1, C2, and C4) to TNF-α/CHX following polyamine depletion and demonstrated that this in-

Fig. 4. Apoptosis response of control and polyamine-deficient IEC-6 cells to TNF-α in combination with CHX in the presence or absence of LY294002. A, TNF-α/CHX-induced apoptosis after various treatments. Cells were grown in cultures containing 5 mM DFMO for 6 days, and LY294002 (LY) at a concentration of 20 μM was given 24 h before exposure to TNF-α (20 ng/ml) + CHX (25 μg/ml). Apoptosis was measured by morphological analysis (middle) and ApoAlert annexin V staining (left) 4 h after the treatment with TNF-α/CHX. a, controls; b, control cells exposed to TNF-α/CHX for 4 h; c, cells treated with DFMO alone; d, DFMO-treated cells exposed to TNF-α/CHX; e, DFMO-treated cells exposed to LY294002 alone; f, DFMO-treated cells pretreated with LY294002 and then exposed to TNF-α/CHX. Original magnification ×150. B, percentage of apoptotic cells after different treatments as described in A. Values are mean ± S.E. of data from three experiments. *, p < 0.05 compared with cells treated without TNF-α/CHX (No-TNF-α/CHX).

Fig. 5. Characterization of stable dominant negative mutant Akt (DNMAkt)-transfected IEC cells. A, structure of DNMAkt expression vector. The complete open reading frame (ORF) of DNMAkt cDNA containing a Myc tag at the 3' end of the Akt ORF was cloned to an expression pUSEamp(+) vector. PCMV, human cytomegalovirus immediate-early promoter. B, representative autoradiograms of Western blot for Myc tag and p-Akt proteins. IEC-6 cells were transfected with the expression vector containing either DNMAkt cDNA or wild-type Akt (WT-Akt) cDNA by the LipofectAMINE technique, and clones resistant to the selection medium containing 0.6 mg/ml G418 were isolated and screened for DNMAkt or WT-Akt expression by Western blot analysis. Whole cell lysates from each of clones (C) and the cells transfected with the control vector were harvested, and levels of Myc tag and p-Akt proteins were measured by Western blot analysis. Actin immunoblotting was performed as an internal control for equal loading. Three separate experiments were performed that showed similar results.
creased resistance to the apoptosis were completely prevented in these independently transfected clones (data not shown). Together, these findings clearly indicate that activated Akt mediates suppression of apoptosis in intestinal epithelial cells following polyamine depletion.

**Effect of Akt Inhibition on Caspase-3 Activation in Polyamine-deficient Cells**—To determine the possibility that activated Akt mediates the suppression of TNF-α/CHX-induced apoptosis by down-regulation of caspase-3 activity, we examined the effect of inactivation of Akt by either treatment with LY294002 or DNMAkt transfection on formation of the active form caspase-3 and its downstream targets PARP and DFFI in polyamine-deficient cells. In control cells, exposure to TNF-α/CHX significantly increased the levels of active caspase-3, PARP, and DFFI proteins (Fig. 8, left). On the other hand, increased Akt activation in DFMO-treated cells was paralleled by a decrease in cleavage of procaspase-3, pro-PARP, and pro-DFFI to their active forms after treatment with TNF-α/CHX. Polyamine depletion did not alter levels of procaspase-3, pro-PARP, and pro-DFFI proteins. Pretreatment of DFMO-treated cells with LY294002 at a concentration of 20 μM for 24 h restored activation of caspase-3, PARP, and DFFI. Consistently, when stable DNMAkt-transfected IEC cells were grown in the presence of DFMO for 6 days, levels of caspase-3, PARP, and DFFI proteins increased significantly as those observed in control cells after exposure to TNF-α/CHX (Fig. 8, right). In addition, putrescine given together with DFMO also restored activation of caspase-3, PARP, and DFFI to near normal levels. These results indicate that polyamine depletion-induced Akt activation inhibits caspase-3 activation in intestinal epithelial cells.

**Effect of Akt Inactivation on Phosphorylation of GSK-3 in Polyamine-depleted Cells**—To determine the possible down-
performed that showed similar results. An internal control for equal loading. Three separate experiments were

DFFI (specific antibodies. Actin (20 ng/ml) and CHX (25 μg/ml)) for 4 h. Whole cell lysates were harvested, applied to each lane (20 μg) equally, and levels of procaspase-3 (~32 kDa), caspase-3 (~17 kDa), pro-PARP (~120 kDa), PARP (~85 kDa), pro-DFFI (~45 kDa), and DFFI (~20 kDa) were identified by probing nitrocellulose with various specific antibodies. Actin (~42 kDa) immunoblotting was performed as an internal control for equal loading. Three separate experiments were performed that showed similar results.

stream signaling of activated Akt in polyamine-deficient cells, several potential Akt target proteins, GSK-3, FKHR, and Bad, were measured after treatment with LY294002 or DNMAkt transfection. As shown in Fig. 9, polyamine depletion by DFMO increased levels of pGSK-3 but had no effect on pFKHR and pBad proteins. The level of pGSK-3 in cells treated with DFMO for 6 days was ~3.8 times the control values. Inhibition of Akt activation by either treatment with LY294002 at the concentration of 20 μM or stable DNMAkt transfection completely prevented the increased levels of pGSK-3 in polyamine-deficient cells. These results suggest that polyamine depletion-induced Akt inhibits caspase-3 activation through a process involving GSK-3 phosphorylation in normal intestinal epithelial cells.

DISCUSSION

As pointed out in the Introduction, cellular polyamines have been intimately implicated in the regulation of apoptosis. Although few specific functions of polyamines in the apoptotic pathway have been defined, there is no doubt that polyamines modulate apoptosis in intestinal epithelial cells through multiple signaling pathways. For example, we (31, 40) and others (42) have recently demonstrated that polyamines regulate NF-κB activity in intestinal epithelial cells and that depletion of cellular polyamines increases the basal levels of NF-κB proteins, induces NF-κB nuclear translocation, and activated its sequence-specific DNA binding activity. The induced NF-κB stimulates the expression of inhibitor of apoptosis proteins, contributing to the inhibition of apoptosis in polyamine-deficient cells. Although it is well known that Akt functions as an important cell survival signal, the involvement of Akt in polyamine-modulated apoptosis has not been established in normal intestinal epithelial cells. The present studies provide new evidence that polyamines are needed for the inhibition of Akt signaling, as depletion of cellular polyamines by treatment with DFMO induced Akt phosphorylation (Fig. 1) and increased its kinase activity in IEC-6 cells (Fig. 2). Increased Akt phosphorylation following polyamine depletion is specific, because there were no significant changes in levels of phosphorylated ERK and p38 proteins in DFMO-treated cells.

The results presented in Fig. 3 clearly show that blocking the activity of PI3K by its specific inhibitor, LY294002, abolished the increased phosphorylation of Akt in DFMO-treated cells, suggesting that polyamine depletion induces Akt activation primarily through the PI3K-dependent signaling pathway in intestinal epithelial cells. A series of studies has suggested that PI3K is necessary and sufficient for activation of Akt in response to certain extracellular and intracellular stimuli in a variety of cell types. Treatment with the specific PI3K inhibitors prevents activation of Akt, indicating that PI3K is an upstream regulator of Akt (43). Dominant inhibitory alleles of PI3K block Akt activation (18, 43), whereas constitutively activated PI3K increases Akt activity independent of stimulation
of extracellular factors such as growth factors (21, 44). Furthermore, pretreatment with the specific PI3K inhibitor prevents activation of Akt by growth factors (45). It has been shown that Akt molecules are able to dimerize and interact with other proteins through the N-terminal region that includes a pleckstrin homology domain (17, 18). Homooligomerization of Akt is induced by the products of PI3K, PtdIns(3,4,5)P3, and PtdIns(3,4)P2, which trigger the simultaneous phosphorylation of Akt by phosphatidylinositol-dependent kinase 1 and 2. Phosphatidylinositol-dependent kinase 1 phosphorylates Thr308 of Akt, whereas the phosphorylation of Ser473 is independent of Thr308 (46). Although Ser473 is phosphorylated by a kinase whose identity is still obscure, it has been proposed as phosphatidylinositol-dependent kinase 2. In addition, the other possibility has been proposed, suggesting that autophosphorylation at the Ser473 site is the mechanism by which this serine residue is phosphorylated (47).

The most important findings reported in this article are that Akt plays a critical role in the regulation of apoptosis by polyamines in normal intestinal epithelial cells. The activated Akt was associated with an increased resistance to TNF-α/CHX-induced apoptosis in polyamine-deficient IEC-6 cells, whereas inactivation of Akt by either treatment with LY294002 (Fig. 4) or ectopic expression of dominant negative Akt mutant (Fig. 7) prevented this tolerance to cell death. Furthermore, normal stable DNMAkt-transfected IEC-6 cells (without DFMO) that were paralleled by a significant decrease in phosphorylation of Akt (Fig. 5) exhibited the increased susceptibility to apoptosis after exposure to TNF-α/CHX (Fig. 6). These findings are consistent with results from others (24–27, 48), who have demonstrated that Akt activation suppresses apoptosis induced by different death stimuli in a variety of cell types. It has been shown that overexpression of wild-type Akt protein prevents apoptosis in primary cultures of cerebellar neurons that are induced by survival factor withdrawal or inhibition of PI3K (23). In contrast, the expression of dominant negative Akt mutants interferes with growth factor-mediated survival in these cells, indicating that Akt is essential for neuronal survival (21). In other systems, overexpression of constitutively activated Akt blocks UV-induced apoptosis in Raf-1 and COS-7 cells and prevents cell death that induced detachment of Madin-Darby canine kidney cells from their extracellular matrix (49). Moorehead et al. (27) have also reported that sustained phosphorylation of Akt inhibits mammary epithelial apoptosis in mouse mammary tumor virus-insulin-like growth factor II transgenic mice.

Observations from the current studies further indicate that activation of Akt following polyamine depletion rescues IEC-6 cells from TNF-α/CHX-induced apoptosis by inhibiting caspase-3 activity. Caspases, a group of cysteine proteases, are the most extensively investigated activators of apoptosis and directly execute the death program (50, 51, 53). To date, 14 different caspases ranging in size from 17 to 55 kDa have been identified in mammals (52, 53). Of these, the caspase-3 is a short proarm caspase that is mostly activated through the action of initiator caspases to mediate irreversible cell damage leading to apoptosis. In response to the apoptotic stress, pro-caspase-3 is cleaved to the active form of caspase-3 that activates its downstream target proteins such as PARP and DFFI. As shown in Fig. 8, activation of Akt in polyamine-deficient cells was associated with a significant decrease in levels of active caspase-3, PARP, and DFFI proteins after exposure to TNF-α/CHX. Inhibition of Akt activity by pretreatment with LY294002 or expression of DNMAkt not only prevented the suppression of caspase-3 activation in polyamine-deficient cells but also returned activation of PARP and DFFI to near normal. These findings are not surprising, because several studies have indicated that polyamine depletion inhibits activation of caspases through multiple signaling pathways. It has been reported that N-terminal c-Jun kinase is implicated in the activation of caspase in response to TNF-α/CHX in intestinal epithelial cells and polyamine depletion prevents the activation of the N-terminal c-Jun kinase and caspases-3, -6, -8, and -9 (54). Polyamines are also shown to modulate the caspase activity by altering ERK phosphorylation in transformed mouse fibroblasts (55, 56). We have recently found that inhibition of caspase-3 activation results partially from the induction of the NF-κB-mediated inhibitor of apoptosis protein expression following polyamine depletion (40).

The exact mechanism through which increased Akt inhibits activation of caspase-3 is still obscure, but may be related to the stimulation of GSK-3 phosphorylation in polyamine-deficient cells. Results presented in Fig. 9 indicate that polyamine depletion significantly increased levels of pGSK-3, which was completely prevented by inhibition of Akt activation through treatment with LY294002 or expression of DNMAkt. The Akt-induced GSK phosphorylation in polyamine-deficient cells is specific because there were no changes in levels of pFKHR and pBad proteins. Our study is consistent with previous work that has demonstrated that down-regulation of Akt and the consequent failure to phosphorylate GSK-3 cause apoptosis (57). Suhara et al. (24) have recently found that constitutive activation of Akt increases GSK-3 phosphorylation, inhibits caspase-3 activity, and prevents β-amyloid-induced apoptosis in endothelial cells. It has been shown that GSK is an Akt substrate and its kinase activity is regulated upon serine phosphorylation by Akt (58). GSK-3 is implicated in the regulation of multiple physiological processes by phosphorylation of a broad range of substrates including numerous transcription factors such as AP-1, c-Myc, and c-Myb and the translation factor eIF2B (59, 60). Our previous studies have demonstrated that polyamine depletion increases JunD/AP-1 activity and that increased JunD/AP-1 is functionally significant in intestinal epithelial cells (9, 61). It is not clear at present that activated Akt/GSK-3 is able to interact with JunD/AP-1 signaling and cooperatively modulate caspase-3 activation in polyamine-deficient cells. Clearly, further studies are needed to determine how the induced Akt/GSK-3 signaling pathway mediates inhibition of caspase-3 activation and apoptosis following polyamine depletion.

In summary, these results indicate that polyamine depletion induces the Akt activation, which plays a critical role in the increased resistance to TNF-α/CHX-induced apoptosis in normal intestinal epithelial cells. The increased Akt in polyamine-deficient cells is mediated through the PI3K-dependent pathway and the resultant activation of Akt induces phosphorylation of its downstream target proteins such as GSK-3. Inactivation of Akt by either treatment with LY294002 or ectopic expression of a DNMAkt prevented the increased resistance to TNF-α/CHX-induced apoptosis in polyamine-deficient cells. The present study also shows that activated Akt mediates suppression of apoptosis at least partially through inhibition of caspase-3. These findings suggest that Akt kinase is an important cell survival factor in the gut mucosa in vivo and plays an important role in biological regulation of apoptosis in normal intestinal epithelial cells. Polyamines are implicated in the maintenance of intestinal epithelial integrity in association with their ability to regulate apoptosis by altering Akt signaling pathway.

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