Insulin-like growth factors are more effective than progastrin in reversing proapoptotic effects of curcumin: critical role of p38MAPK

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Singh P, Sarkar S, Umar S, Rengifo-Cam W, Singh AP, Wood TG. Insulin-like growth factors are more effective than progastrin in reversing proapoptotic effects of curcumin: critical role of p38MAPK. Am J Physiol Gastrointest Liver Physiol 298: G551–G562, 2010. First published February 4, 2010; doi:10.1152/ajpgi.00497.2009.—Progastrin and insulin-like growth factors (IGFs) stimulate hyperplication of intestinal epithelial cells (IECs) via endocrine/paracrine routes; hyperplication is a known risk factor for colon carcinogenesis. In the present study, inhibitory potency of curcumin in the presence or absence of progastrin and/or IGF-II was examined. Progastrin and IGF-II significantly increased proliferation of an immortalized IEC clone line, IEC-18, whereas curcumin decreased the proliferation in a dose-dependent manner. IGF-II was significantly more effective than progastrin in reversing antiproliferative effects of curcumin and reversed proapoptotic effects of curcumin by >80%; progastrin was relatively ineffective toward reversing proapoptotic effects of curcumin. IEC-18 clones were generated to overexpress either progastrin (IEC-PG) or hIGF-II (IEC-IGF). Proliferation of IEC-PG and IEC-IGF clones was increased, compared with that of control clones. Curcumin significantly reduced proliferation of IEC-PG, but not IEC-IGF, clones. Similarly, a human colon cancer cell line, Caco-2 (which expresses autocrine IGF-II), was relatively resistant to inhibitory effects of curcumin. However, Caco-2 cells treated with anti-IGF-II-antibodies were rendered sensitive to inhibitory effects of curcumin. Significant differences in inhibitory potency of curcumin against PG- vs. IGF-II-stimulated growth of IEC-18 cells were not reflected by differences in curcumin-mediated inhibition of activated (phosphorylated) ERKs/IKK and c-Src in wild-type (wt) IEC-18 cells, in response to the two growth factors. Surprisingly, curcumin was almost ineffective in reducing IGF-II-stimulated activation of p38MAPK but significantly reduced progastrin-stimulated phosphorylation of p38. Treatment with a p38MAPK inhibitor resulted in loss of protective effects of IGF-II against inhibitory effects of curcumin. These novel findings suggest that growth factor profile of patients and tumors may dictate inhibitory potency of curcumin and that combination of curcumin + p38MAPK inhibitor may be required for reducing hyperplications or tumorigenic response of IECs to endocrine and autocrine IGFs.

NF-κB; c-Src; IEC-18 cells; Caco-2 cells

COLORECTAL CANCERS (CRCs) are one of the most common forms of cancers in men and women in the US and are one of the leading causes of death. Although genetic instability plays a dominant role in familial cancers, hyperplication, in response to aberrant growth factor signaling, is believed to play a permissive role in initiation and progression of sporadic cancers. Several growth factors, including progastrins (PG) and insulin-like growth factors (IGFs), exert potent proliferative effects on normal and cancerous intestinal cells in vitro (8, 31, 39). Studies with mutant mouse models suggest that PG and IGFs function as cocarcinogens and increase the risk of colon carcinogenesis or tumorigenesis (5, 7, 40, 51). Experimental evidence further suggests that PG and IGFs exert growth promoting effects during all phases of colon carcinogenesis via endocrine/paracrine/autocrine routes (5, 7, 8, 39, 40, 51).

The full-length, 80-amino-acid PG peptide is normally expressed in gut enterodocrine cells in the stomach and processed by endopeptidases into G-Gly; subsequent amidation at carboxy terminus generates amidated gastrins (G34, G17). Under physiological conditions, only amidated gastrins are present in circulation and play a role in meal-stimulated acid responses. However, in patients with hypergastrinemia and CRCs, significant levels of circulating PG and G-Gly are detected (reviewed in Ref. 31). Azoxy methane induced colonic tumors in rats, and a significant percent of human colon tumors express gastrin gene (31). However, colon cancer cells lack the ability to process PGs (46), and thus elevated levels of circulating PG are measured in rats and humans bearing colonic cancers (4, 31, 35). Importantly, autocrine gastrins (mainly PG) are required for maintaining tumorigenic potential of gastrin-dependent colon cancers (13, 38).

Epidemiological and experimental studies suggest a correlation between circulating levels of free IGF-I and relative risk for developing colon, breast, prostate, and lung cancer (22, 33). Autocrine IGFs (mainly IGF-II) play an equally important role in conferring a growth advantage to many cancer cells (8, 36, 39). IGF-II expression is 10- to 40-fold higher in CRC than in normal colonocytes (8, 39). IGF-II expression is 10- to 40-fold higher in CRC com- pared with that in normal colonocytes (8, 39). Thus both circulating and autocrine/paracrine IGFs and PG stimulate hyperplication of colonic crypt cells and exert potent co- carcinogenic effects at all phases of colon carcinogenesis.

Curcumin [diferuloylmethane; 1,7-bis-(4-hydroxy-3-methoxy-phenyl)-1,6-heptadiene-3,5-dione] is the major pigment in turmeric powder and has anti-inflammatory and antioxidant properties (12, 19). Curcumin inhibits chemically induced carcinogenesis during initiation and/or postinitiation phases (27). Chemopreventive activity of curcumin was also demonstrated during the promotion and progression phases of colon carcinogenesis (17). Mechanisms by which dietary curcumin exerts chemopreventive effects at different phases of colon carcinogenesis were recently reviewed (34). Although antiproliferative effects of curcumin have been observed on most colon cancer cell lines, apoptotic effects of curcumin have not always been observed (3, 10). These differences may reflect growth factor profiles of CRC cells. Overexpression of
specific antiapoptotic factors and signaling molecules such as Hsp70 (29), Bcl-xL, and ku70 (28) reduce inhibitory efficacy of curcumin on cancer cells. Thus elevated levels of endocrine/autocrine IGF-II and PG (relevant to colon cancer disease) can be expected to potentially impact chemopreventive efficacy of curcumin. To examine inhibitory effectiveness of curcumin in the presence of either PG or IGF-II, it was critical that we use a cell line that is responsive to IGF-II or PG but does not express the two growth factors so that the results are compared within an isogenic background. Almost all colon cancer cells express either the gastrin gene (PG) and/or IGF-II; for example, HCT-116 cells express gastrin gene and Caco-2 cells express autocrine IGF-II. Although HCT-116 cells are responsive to exogenous IGF-II, they do not respond to exogenous PG. Caco-2 cells become generally nonresponsive by day 9 and undergo spontaneous differentiation in culture by days 9–12. It is thus difficult to develop an isogenic background of cancer cells that are equally responsive to endocrine/autocrine IGF-II AND PG. In the present studies, we examined inhibitory efficacy of curcumin on an immortalized intestinal cell line (IEC-18), which is responsive to both growth factors and does not express IGF-II AND PG. Our hypothesis is that if hyperproliferative effects of growth factors are attenuated in the presence of dietary agents, such as curcumin, then the risk associated with hyperproliferation toward neoplastic transformation of colonic crypts, in the presence of initiating agents (such as DNA-damaging agents), can be significantly reduced. Thus, to address our major hypothesis, we chose the nontransformed IEC-18 cell line, which reflects the phenotype of colonic crypt cells in the proliferative zone.

Inhibitory potency of curcumin was also examined on Caco-2 cells, which expresses autocrine IGF-II in a density-dependent manner, from days 3–9 of cell culture (36), and represents an ideal cancer cell model for examining role of autocrine IGF-II. We report for the first time that proapoptotic potency of curcumin was almost completely reversed by IGF-II, whereas PG was much less effective, suggesting that elevated endocrine/autocrine IGF-II in cancer patients will likely impart a resistant phenotype to curcumin treatment. To examine mechanisms contributing to observed differences in protective effects of IGF-II vs. PG, phosphorylation (activation) of specific kinases and transcription factors in response to curcumin ± PG and/or IGF-II was examined. Our studies suggest that increased phosphorylation or activation of p38MAPK may contribute to significant differences in protective potency of IGF-II vs. PG against proapoptotic effects of curcumin. These novel findings can be expected to impact clinical use of curcumin in either preventing the transformation and neoplastic growth of colonic crypt cells and/or treating CRCs (and perhaps other epithelial cancers).

**MATERIALS AND METHODS**

**Materials.** Leupeptin, aprotinin, benzamidine, phenylmethylsulfonyl fluoride (PMSF), sodium orthovanadate, ethylenediaminetetraacetic acid (EDTA), Nonindet P-40, octyl-d-glucoside (ODG), β-mercaptoethanol, Tris(hydroxymethyl)aminomethane, HEPES, sodium chloride, sodium fluoride, glyceraldehyde, and campothecin were obtained from Sigma Chemical (St. Louis, MO). Polyclonal anti-active caspase 3 and anti-caspase 9 antibodies were purchased from BD Pharmingen (San Diego, CA); polyclonal anti-β-actin was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal anti-phospho-p65 NF-κB (Ser536), anti-phospho-IκBα (Ser32/36), anti-IκKα/β (Ser176/180), anti-phospho-p44/42 MAP kinase, anti-phospho-P38 MAP kinase, antibodies were from Cell Signaling Technology (Beverly, MA). Anti-v-Src mouse monoclonal antibody was purchased from Calbiochem (La Jolla, CA). IGF-II was purchased from Biosource (San Jose, CA), and rhPG was generated and purified in our laboratory as described (37). Specific anti-PG-Abs were generated in our laboratory as described (5, 32). NF-κB DNA binding assay kit was purchased from Active Motif (Carlsbad, CA). Anti-IGF-II-antibody was purchased either from Santa Cruz (SC1415) or from Abcam (ab63984).

**Cell culture.** IEC-18 cells, a nontransformed intestinal crypt cell line derived from rat ileum (American Type Culture Collection, Rockville, MD) was propagated in DMEM (GIBCO-BRL, Grand Island, NY), supplemented with 10% heat-inactivated fetal calf serum (FCS, Hyclone, Logan, UT), 4 μM L-glutamine, 0.1 μM nonessential amino acids, 1 μM sodium pyruvate, 100 units/ml penicillin G sodium, and 100 mg/ml streptomycin sulfate in an atmosphere of 95% air-5% CO2 at 37°C as described previously (37). Caco-2 cells, a human colon cancer cell line, originally obtained from Dr. Jing Yu, Tufts School of Medicine (Boston, MA), has been maintained in our laboratory at early passages (16–35) for several years. Caco-2 cells were maintained in cell culture as described previously (36). The cell lines were regularly monitored for the absence of mycoplasma, by using a Mycoplasma Detection Kit (Boehringer Mannheim), and confirmed to be positive for E-cadherin. Stock cultures of cells were subcultured at appropriate intervals to maintain the cells at subconfluent densities. For cell counting and subculturing, the cells were dispersed with a solution of 0.05% trypsin and 0.02% EDTA.

**Generation of IEC-18 clones expressing either hPG or hIGF-II.** Eukaryotic expression plasmids were created for expression of full-length coding sequences either for triple-double mutant (3×) hhGAS genes (R57A-R58A, K74A-K75A, R94A-R95A) (as previously described; Ref. 5) or for human insulin-like growth factor II (IGF-II) gene, using a modified pcDNA3.1 vector (Invitrogen). An ATF, two serine, and six histidine codons were inserted 3′ to the vector’s HindIII site. The histidine sequence is followed by an Xhol and a BamHI restriction site. Target inserts for 3× mutant hhGAS and the hIGF-II open reading frame (accession no. J03242.1) were PCR amplified with primers that introduced a 5′ Xhol site and a 3′ BamHI site (post the termination codon) into the amplified DNA; the previously described Fabp-mut hGAS plasmid (5) was used as a template for amplifying mut hGAS cDNA. hGAS II cDNA from a human colon cancer cell line (Caco-2) was used as a template for amplifying the IGF-II transcript. Final constructs were confirmed by DNA sequence analysis. The plasmids were transfected into the IEC-18 cells to create stably expressing clones that expressed either IGF-II (IEC-IGF-II) or PG (IEC-PG) by our published methods (38, 41). The vector-transfected clones were used as a control (IEC-C). The clones were confirmed for the expression of PG and IGF-II by Western blot and dot-blot analysis (data not shown), as described previously (5, 36). The concentration of PG and IGF-II secreted by the clones into the conditioned medium of the cells/24 h was determined as described previously (36, 38) to be ~100–150 pg/ml/1 × 10⁵ cells (which is equivalent to 1–2 nM peptide).

**In vitro growth assays.** The effect of increasing concentrations of curcumin, in the presence or absence of PG or IGF-II, on the growth response of IEC-18 cells was measured either in a cell count assay or as a 3[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay as previously described (37). Briefly, for cell count assay, an optimal number of cells (3.5 × 10⁵ cells) were plated in 35-mm dishes in 2 ml of growth medium containing 10% FCS. After 24 h, medium was changed to serum-free medium (SFM) for 72 h, followed by treatment of the cells with increasing concentrations of growth factors for 24 h in SFM, after which increasing concentrations of curcumin were added (~10–100 μM) and incubated for 24–48 h. At the end of the treatment, cells were treated with trypsin-EDTA solution and counted via a Multisizer3 Coulter electronic counter.
(Beckman Coulter, Fullerton, CA). To measure the effect of growth factors on the antiproliferative effects of curcumin, MTT assay was also used as described previously (37). Briefly, an optimal number of cells (4.5 × 10^4) were plated in 96-well plates in 200 μl of growth medium containing 10% FCS. After 24 h, the medium was changed to SFM for 72 h, followed by addition of PG or IGF-II for 48 h; curcumin was then added and incubated for additional 24–48 h. Cells were then processed for the MTT assay (37). Inhibitory efficacy of curcumin was also examined on the growth of Caco-2 cells in culture, in the presence or absence of anti-IGF-II-antibodies as described in the legend of Fig. 2B.

Treatment of IEC-18 cells for measuring surrogate markers of apoptosis. Cells were cultured in either 35-mm dishes or T-75 flasks (4 × 10^5), essentially as described above. For measuring surrogate markers of apoptosis (relative levels of activated caspases 3 and 9), cells were additionally treated for 4 h with the proapoptotic agent (camptothecin), as previously described (49). At the end of the treatment, cells were washed twice with ice-cold PBS, followed by cellular lysis with either ODG buffer (50 μM Tris-HCl, pH 8.0, 1 μM EDTA, 0.5 M NaCl, 20 μM NaF, 1 μM Na3VO4, 1% Nonidet P-40, 2% octyl-β-glucoside, 5 μM, β-mercaptoethanol, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 μM PMSF, 5% glycerol) or M-PER mammary protein extraction reagent (Pierce) with protease inhibitor cocktail (Sigma). Relative levels of activated caspases 3 and 9 were measured by Western immunoblot analysis as described previously (49).

Western blot analysis. Cellular lysates and cytoplasmic/nuclear extracts were prepared from control or treated IEC-18 cells as described above, followed by boiling in Laemmli buffer, as described previously (32, 49). Samples were processed for Western immunoblot analysis by our published methods (32). Briefly, equal amounts of protein (40–60 μg) were separated by electrophoresis on either 1% SDS, 10% polyacrylamide gels or NuPAGE 4–12% Bis-Tris Gel (Invitrogen). Protein was transferred electrophoretically to Hybond-ECL nitrocellulose membranes (Amersham Biosciences) and blocked in 10 mM Tris-buffered saline containing 0.1% Tween 20, and 5% (wt/vol) nonfat dry milk for 2 h at room temperature. Complete transfer of proteins was confirmed by GelCode Blue staining (Pierce). Membranes were processed by Western immunoblot analysis, using one or more of the indicated specific primary antibodies for 1 h at 4°C at a dilution of 1:1000. Membranes were washed 4X in Tris-buffered saline containing 0.1% Tween 20, followed by incubation with the appropriate peroxidase-conjugated antibody. The antigen-antibody complexes were detected using chemiluminescence reagent kit (Amersham Pharmacia Biotech) according to the manufacturer’s recommendations. To confirm equivalent loading, membranes were stripped with either 2 M glycine pH 2.8 for 30 min or with strip buffer (Pierce) and were reprobed with either anti-β-actin Ab or an Ab that detects the total amount of the indicated kinase, as internal controls. The relative density of the bands was densitometrically analyzed with the Documentation Analysis System (Model Alphalmager 2.000, Alpha Innotech, San Leandro, CA). The data were calculated as a ratio of the densitometric readings for the protein of interest vs. the appropriate control protein. The ratios obtained for control (no peptide or Ab treatment) samples were arbitrarily assigned a 100% value. The ratios obtained with all other treatments were calculated as a ratio of the densitometric readings for the protein of interest vs. the appropriate control protein. The ratios obtained for all experiments, maximally effective doses of PG (1.0 nM) and IGF-II (10 nM) were used. Both PG and IGF-II significantly reversed growth-inhibitory effects of curcumin, at all doses examined; IGF-II was significantly more effective than PG (Fig. 1, C and D). In an MTT assay, relative protective efficacy of IGF-II vs. PG, against inhibitory effects of curcumin, was further amplified (Fig. 1, E and F). Although the pattern of response measured in a cell count (Fig. 1, C and D) and an MTT (Fig. 1E) assay was similar, the MTT assay was more sensitive.

Inhibitory effects of curcumin on growth of IEC-18 clones, overexpressing either hPG or hIGF-II. IEC-18 clones overexpressing either triple mutant hGAS or wild-type (wt) hIGF-II transcripts were generated and confirmed as described under MATERIALS AND METHODS. Progastrin, transcribed from mutant hGAS transcript, lacks dibasic sites required for processing (5), and full-length PG (80 amino acids) is expressed by IEC-PG clones. All clones were confirmed at RNA and protein levels, as described in MATERIALS AND METHODS. Growth response of clones to increasing concentrations of FCS, in presence or absence of 25 μM curcumin, was examined in an MTT assay. Growth response of IEC-C clones to 5% FCS was arbitrarily assigned a 100% value to normalize values from different experiments. Curcumin significantly inhibited growth of IEC-C clones in response to increasing concentration of FCS; in the absence of FCS, no significant effects were measured (Fig. 2A, Table 1). Growth of IEC-PG and IEC-IGF-II clones increased significantly in response to FCS in a dose-dependent manner (Fig. 2A, Table 1). Curcumin effectively inhibited growth effects of autocrine PG (IEC-PG clones), at all doses of FCS; however, curcumin was not as effective in inhibiting growth effects of autocrine IGF-II (Fig. 2A, Table 1). These results
provide strong evidence that curcumin is significantly more effective in inhibiting growth effects of autocrine PG than autocrine IGF-II, similar to results obtained with exogenous growth factors (Fig. 1).

Inhibitory effects of curcumin on the growth of Caco-2 cells, in the presence or absence of anti-IGF-II antibody. We and others have previously reported that Caco-2 cells express high concentrations of autocrine IGF-II under subconfluent conditions from days 2–5 of cell culture and that the expression or secretion of IGF-II is progressively decreased to negligible levels by day 9 of cell culture, as the cells become confluent (36). In preliminary studies we further reported that subconfluent Caco-2 cells on days 3–5 of cell culture (expressing relatively high levels of IGF-II) were resistant to proapoptotic and growth inhibitory effects of curcumin, whereas Caco-2 cells on days 7–9 of cell culture (expressing relatively low levels of IGF-II) were sensitive to inhibitory effects of curcumin (26). In the present studies we further examined the role of autocrine IGF-II in impacting the sensitivity of Caco-2 cells to the inhibitory effects of curcumin. Day 5 Caco-2 cells were treated with either nonimmune (control) IgG or specific anti-IGF-II-IgG for 2 h, followed by curcumin treatment as described in the legend of Fig. 2B. Anti-IGF-II-Abs, by itself, reduced the growth of Caco-2 cells (data not shown) as reported previously (36), confirming an important role of endogenous IGF-II in maintaining proliferation of Caco-2 cells. The growth of Caco-2 cells, growing in response to 1% FCS, was inhibited by ~50%, on treatment with 25 µM curcumin (Fig. 2B); curcumin may have primarily inhibited growth in response FCS. Importantly, Caco-2 cells treated with anti-IGF-II-IgG were further sensitized to the inhibitory effects of curcumin by ~30% (10 µM) to ~70% (25 µM), resulting in almost complete attenuation of growth (25 µM) (Fig. 2B). Growth of cells treated with nonimmune control IgG remained similar to that of cells treated with curcumin alone (Fig. 2B). Thus results with Caco-2 cells resemble the results with IEC-IGF-II clones (as described above) and further confirm the novel paradigm that autocrine IGF-II expression imparts a resistant phenotype to inhibitory effects of curcumin.

Effect of curcumin ± growth factors on apoptotic response of IEC-18 cells to camptothecin. Curcumin significantly increased apoptotic response of IEC-18 cells to camptothecin, in a dose-dependent manner; antiapoptotic effects of PG and IGF-II were confirmed (Fig. 2C). PG and IGF-II reduced camptothecin-induced apoptosis of IEC-18 cells by ~50% (PG) and 60–70% (IGF-II) (Fig. 2C). Both PG and IGF-II significantly reversed (reduced) proapoptotic effects of 10–25 µM curcumin (Fig. 2, D and E); PG was relatively ineffective against 50–100 µM curcumin, whereas IGF-II remained significantly effective (Fig. 2F). In summary, IGF-II was significantly more effective than PG in reversing antiproliferative and proapoptotic effects of curcumin (Fig. 1, F and E).

Curcumin significantly increased activation of caspase 3 (Fig. 3A). Sensitivity of cells to proapoptotic effects of curcumin were enhanced in cells that were 50% (Fig. 3A) vs. 70% (Fig. 3B) confluent. Thus confluence further impacts degree of

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*Fig. 1. Effect of curcumin (Cur) ± IGF-II or progastrin (PG) on growth of IEC-18 cells (cell count assay). IEC-18 cells were treated with curcumin ± IGF-II or PG as described in MATERIALS AND METHODS, and the effect was assessed in a cell count assay. Data are presented as means ± SE of 4 values from a representative of 3 experiments. *P < 0.05 vs. control values (no treatment) in A and B; †P < 0.05 vs. indicated bars in C and D. E: effect of curcumin ± IFI-II/PG on growth of IEC-18 cells (MTT assay). Absorbance measured in control wells (no treatment) was arbitrarily assigned a 100% value. Readings from all other wells are expressed as a % change from control values. Each bar represents mean ± SE of 4 values from a representative of 3 experiments. *P < 0.05 vs. control; †P < 0.05 vs. curcumin alone. F: percent reversal of antiproliferative effects of curcumin, in the presence of growth factors. The % reversal of antiproliferative effects of curcumin by growth factors in an MTT assay (E), to levels measured in the presence of growth factors alone are shown. Values measured in the presence of growth factors alone were arbitrarily assigned 100%. Values presented for growth factor + curcumin are presented as a % of growth factor alone. *P < 0.05 vs. the corresponding values for PG + curcumin.
response of cells. Protein yield from 50% confluent cells (Fig. 3C) was low and lysates from several experiments had to be pooled. Pattern of response, however, was similar in 50% (Fig. 3, A and C) and ~70% (Fig. 3, B and D) confluent cells. IGF-II was ~50–300% more effective than PG in reversing activation of caspase 3 (Fig. 3, A and B) and caspase 9 (Fig. 3, C and D), in response to curcumin. Results with caspase 9 activation (Fig. 3, C and D) agreed with cell death assay results in Fig. 2D. Differences in the effects on activation of caspases 3 vs. 9 may reflect differences in sensitivity of the two antibodies. Cells
undergoing apoptosis detach from extracellular matrix and float in cell culture medium, as an indirect reflection of cell death (49). Numbers of floating cells significantly increased in response to curcumin and significantly decreased in response to PG/IGF-II; IGF-II was significantly more effective than PG in reversing apoptotic effects of PG in the floating assay (Fig. 3E). Average percentage(s) by which growth factors attenuated proapoptotic effects of curcumin are shown in parenthesis above the relevant bar graphs; proapoptotic effects of curcumin were reversed by \( \text{IGF-II} \) – 70 – 85% by IGF-II and by only 25–30% by PG (Fig. 3).

Effect of curcumin + growth factors on phosphorylation of \( \text{IKK}/\beta/\text{NF-κBp65} \).

An optimal dose of curcumin (25 \( \mu \)M) was chosen, since at this dose both PG and IGF-II demonstrated...
strated significant protective effects; at higher doses only IGF-II was protective (Fig. 2D). Representative Western blot data are presented in top panels of Fig. 4, A and B. Bar graphs represent means ± SE of data from four blots from two separate experiments, and are a ratio of the indicated phospho-(p) form of kinase molecule to levels of total kinase (Fig. 4A)/β-actin (Fig. 4B) in the sample. Data in Fig. 4, A and B, are from ~50% confluent cells. Curcumin treatment resulted in significant loss in relative levels of pIKKa/β (Fig. 4A) and pNF-κBp65536 (Fig. 4B); both PG and IGF-II significantly increased levels of pIKKa/β and p65536. Both PG and IGF-II significantly reversed inhibitory effects of curcumin on phosphorylation of p65536; IGF-II was more effective (Fig. 4B). To confirm functional relevance of the observed changes in phosphorylation status of p65, activation of NF-κB was measured in a DNA binding assay, as described previously (32, 44). Change in NF-κB activity was calculated as a percent change from control (nonstimulated) values, wherein NF-κB activity in control samples was assigned a 100% value. Surprisingly PG was more effective than IGF-II in increasing DNA binding (activation) of NF-κB (Fig. 4C). Reason(s) for the apparent discrepancy in relative potency of the two peptides toward increasing relative levels of p65 vs. increasing DNA binding of p65 (in an in vitro assay) is not clear; possible reasons are discussed below. Based on results presented in Fig. 4, it appears that both IGF-II and PG were effective in reversing inhibitory effects of curcumin on phosphorylation and activation of IKKα/β and NF-κBp65. Thus the vast difference in protective effects of IGF-II vs. PG against antiproliferative/proapoptotic effects of curcumin on IEC-18 cells, is not likely mediated via the NF-κB pathway alone. Average percentage by which growth factors reversed inhibitory effects of curcumin on phosphorylation/activation of IKKα/β and NF-κBp65 are presented in parentheses on top of the relevant bar graphs (Fig. 4, A and B).

Effect of curcumin ± growth factors on phosphorylation (activation) of p65 and other kinases (p44,42ERKs/c-Src/ p38MAPK). As discussed above, to further examine the underlying mechanisms responsible for the significant differences in the proapoptotic effects of curcumin against PG- vs. IGF-II-mediated growth of IEC-18 cells, large-scale studies were conducted to analyze relative levels of several additional kinases within one experiment itself (Fig. 5A). In the large-scale studies, T75 flasks of IEC-18 cells were treated with curcumin ± growth factors. Rather than growing the cells to only 50% confluence (Figs. 4, A–C), cells were grown to 70% confluence (Fig. 5A), to obtain larger amounts of cellular lysate protein. A representative Western blot, demonstrating relative levels of p65536, pp65276, ppERK, pc-Src, and ppp38MAPK is presented from one of two similar experiments; relative levels of β-actin in the corresponding samples from a representative β-actin blot are shown in Fig. 5A. The ratio of the indicated phosphorylated kinase to β-actin levels, calculated as a percent of control (as described in the legends of Figs. 4, A and B), from a total of four blots from two experiments, are presented in Figs. 5, B and C, and 6, A–C. Once again the degree of inhibitory (curcumin) and stimulatory (growth factors) responses of IEC-18 cells were significantly enhanced in cells treated at 50% (Fig. 4, A and B) vs. ~70% (Fig. 5, A–C) confluence. In other words, protective effects of growth factors were blunted in 70% confluent cells (Fig. 5A).

Surprisingly, levels of pNF-κBp65276 were also significantly increased by IGF-II, but not PG; IGF-II slightly reversed inhibitory effects of curcumin on pNF-κBp65276 (Fig. 5C). Since IGF-II increased phosphorylation of NF-κB at both Ser536 and Ser276 (Fig. 5, A–C), it may have caused spatial hindrance in an in vitro DNA binding assay (Fig. 4C) and may thus represent an artifact of the in vitro assay, resulting in the observed difference in the DNA binding of PG- vs. IGF-II-stimulated samples. However, both PG and IGF-II significantly
reversed inhibitory effects of curcumin on DNA binding of \( \kappa B \) (Fig. 4C).

In the absence of FCS, curcumin did not significantly reduce activation of ERKs (Figs. 5A and 6A) but significantly reduced levels of \( pS\kappa B \) and \( pp38MAPK \) (Figs. 5A and 6B). Both PG and IGF-II significantly increased phosphorylation of ERKs/c-Src\( ^{416} \)/\( pp38MAPK \) (Figs. 5A and 6A–C); IGF-II was generally more potent than PG, but the differences were not statistically significant. Both PG and IGF-II significantly reversed inhibitory effects of curcumin on phosphorylation of ERKs/c-Src, with almost equal potency (Figs. 5A and 6A and B). Numbers in parentheses, on top of the bar graphs, represent average percentages by which IGF-II AND PG reversed inhibitory effects of curcumin on the indicated kinase(s). The most interesting finding was that although IGF-II overwhelmingly reversed inhibitory effects of curcumin on \( pp38MAPK \) (by \( \approx 90\% \)), PG was almost ineffective (Figs. 5A and 6C). Thus the stark difference in protective effects of IGF-II vs. PG on phosphorylation of \( pp38MAPK \) may have contributed to differential effects of IGF-II vs. PG in overcoming proapoptotic effects of curcumin (Fig. 2D). To test this novel possibility, we used a specific \( pp38MAPK \) inhibitor (SB203580) (Fig. 6D). Inhibition of \( p38 \) overcame protective effects of IGF-II against inhibitory effects of curcumin (Fig. 6D), providing further evidence that IGF-II mediated activation of \( pp38MAPK \) likely contributed to potent protective effects of IGF-II.

**DISCUSSION**

The present studies have led us to discover several new paradigms that can be expected to impact clinical use of curcumin as a chemopreventive agent against intestinal hyperproliferation and tumorigenesis. Our studies strongly suggest that inhibitory efficacy of curcumin will be significantly reduced in the presence of endocrine/autocrine growth factors, especially IGFs. Protective potency of the two physiologically relevant endocrine/autocrine growth factors against inhibitory effects of curcumin was growth factor specific. Protective potency of exogenous/autocrine IGF-II was two- to threefold higher than that of PG against inhibitory effects of curcumin on proliferation/survival of IEC-18 cells in vitro. Similarly, a human colon cancer cell line (Caco-2, which expresses significant levels of autocrine IGF-II on days 3–5 of cell culture; Ref. 36) was resistant to inhibitory effects of curcumin; however, day 5 Caco-2 cells were sensitized to inhibitory effects of curcumin in the presence of anti-IGF-II-antibodies (Fig. 2B).

Results with IEC-18 cells suggested a possible difference in inhibitory potency of curcumin against signaling pathways required for mediating proliferative and antiapoptotic effects of the two growth factors. In the case of PG, \( NF-\kappa B \) activation by the canonical pathway (activation of IKK and degradation of IkBo\( \alpha \)), downstream of \( pp38MAPK/ERK \), is critically required for measuring growth effects of PG in vitro (32) and on colonic crypts in vivo (44). NEMO peptide (selective inhibitor of IKK\( B \)) abrogated antiapoptotic effects of PG in vitro (32) and in vivo (44), suggesting an important role of IKK\( B \) in mediating PG effects. A critical role of NF-\( \kappa B \) in mediating antiapoptotic effects of PG was confirmed in experiments with NF-\( \kappa B\)p\( 65 \) siRNA (32). Nuclear translocation/activation of \( \beta \)-catenin were also measured (in vitro) and in vivo in IECs and colonic crypts of mice, in response to PG (45); parallel findings were reported in cancer cells, downregulated for PG expression (24). Our recent findings suggest that \( \beta \)-catenin activation in response to PG is downstream of NF-\( \kappa B \) activation (45); thus activation of NF-\( \kappa B \) remains critical for measuring proliferative and antiapoptotic effects of PG. Besides PG, other gastrointestinal hormones, such as neurotensin (47) and amidated gastrins (43), also upregulate NF-\( \kappa B \)-mediated regulatory molecules such as IL-8. Thus growth-promoting and antiapoptotic effects of gastrointestinal hormones, via their cognate receptors, can be potentially mediated by the target gene products of activated NF-\( \kappa B \) pathways.

![Fig. 5. A: representative Western blots for activated kinases in response to curcumin ± PG/IGF-II. Data presented is from one of 4 blots from 2 separate experiments. Relative levels of \( \beta \)-actin measured in a representative blot from corresponding samples is shown. B and C: inhibitory effects of curcumin on activation of \( pNF-\kappa Bp65^{\alpha} \) (B) and, \( pNF-\kappa Bp65^{\beta} \) (C) in response to growth factors. Western blot data, as shown in A, were used to calculate the ratios as described in Fig. 4. Briefly, phosphorylated kinase values were calculated at a ratio of \( \beta \)-actin. Ratios of control (untreated) samples were assigned 100% values, and ratios of all other samples were calculated as a % of control. Data in each bar = means ± SE of 4 measurements from 2 experiments. Dotted horizontal lines = inhibitory effects of curcumin on activation of indicated kinases, as described in Fig. 4. Percent activation of kinase by PG/IGF-II was assigned 100% values; numerical values in parentheses = % loss in kinase activation in the presence of curcumin, compared with that in the presence of PG/IGF-II alone. * \( p < 0.05 \) vs. control; † \( p < 0.05 \) vs. curcumin alone.](http://ajpgi.physiology.org/DownloadedFrom/http://ajpgi.physiology.org/)
In the case of IGFs, activation of IGF-I receptor (IGF-I-R) results in recruitment and phosphorylation of adaptor proteins insulin receptor substrate (IRS-I) and SHC (33), which serve as docking sites for other signaling molecules, resulting in activation of several downstream pathways, including c-Src, PI3K/Akt, and p38MAPK/ERKs (33). IGF activation of colon cancer cell lines was shown to protect them from apoptosis by potentiating TNF-α/MAPK/NF-κB signaling pathways (30). Constitutive activation of IGF-I-R in immortalized mammary epithelial cells resulted in overexpression of activated NF-κB and was associated with transformation of the cells (18). Activation of NF-κBp65 was essential for measuring IGF-I-mediated stimulation of metatarsal growth and growth plate chondrogenesis (50). Increased expression of allelic Igf-II increased elongation of intestinal crypts and adenoma growth (11). Results of present studies additionally suggest that IGF-II activates NF-κB in IEC-18 cells (Figs. 4 and 5). Increase in cellular levels of reactive oxygen species (ROS) results in activation of NF-κB, and curcumin reduces cellular levels of ROS (reviewed in Ref. 21). Relative levels of ROS are high in cancer cells but are barely detectable in normal cells. Even though IEC-18 cells are immortalized and can be propagated in the presence of FCS and/or growth factors, the cell line is nontumorigenic. IEC-18 cells have relatively low levels of ROS (unpublished data from our laboratory) and express low levels of activated NF-κB (present study). However, in the presence of PG/IGF-II, significant increases in levels of activated NF-κB were measured (Figs. 4, 5).

Activation of NF-κB is a crucial event both in inflammation and cancer (16). Inhibition of NF-κB plays an important role in curcumin induced apoptosis (reviewed in Ref. 19, 21, 34). Inhibitory effects of curcumin are mediated through IκB/NF-κB pathway in transformed IEC and colon cancer cells (15). Curcumin inhibits cell migration of human colon cancer cells through inhibition of NF-κB/p65, COX-2, and MMP-2 expression (42). Constitutively active NF-κB in melanoma cells plays a central role in cell survival and growth; curcumin selectively inhibited growth of melanoma cells but not normal melanocytes (23). Similarly, constitutive activation of NF-κB has been observed in colorectal cancer cells but not in normal colorectal epithelial cells (discussed in Ref. 20). Radiation resistance of tumor cells, due to activation of NF-κB in...
response to radiation therapy, was reversed by curcumin (20). In summary, curcumin suppresses NF-κB activation and down-regulates expression of NF-κB-regulated gene products involved in survival (Bcl2, Bel-xL, XIAP, and cIAP-1), proliferation (COX2, cyclin D1, and c-myc), angiogenesis (VEGF and IL-8), invasion (MMP-9), and metastasis (ICAM-1, VCAM-1, and ELAM-1) (discussed in Ref. 20). Therefore, in the present studies, we focused on examining whether curcumin differentially inhibits activation of NF-κB in response to IGF-II vs. PG as a means of understanding significant differences in inhibitory potency of curcumin against the two growth factors. Since NF-κB activation is critically required for mediating PG effects, it was expected that curcumin will likely inhibit PG-mediated NF-κB activation and hence growth. In the case of IGFs, inhibitory effects of curcumin on IGF-I-R and IGF-stimulated pathways was recently reported on cancer cells (25, 52). However, anticancer effects of curcumin, independent of IGF-I-R inhibition, have also been reported (2). At the same time, an increase in IGF-I levels has been reported in response to curcumin in diabetic rats (14). Thus inhibitory vs. stimulatory effects of curcumin on IGF system may be specific to the cell or system.

In the present studies, both PG/IGF-II significantly increased phosphorylation/activation of NF-κB (Figs. 4 and 5). However, the upstream pathways mediating increase in phosphorylation of NF-κB were probably different, since PG only stimulated an increase in p65Ser536, whereas IGF-II increased both p65Ser276 and p65Ser536. Despite these differences, increases in relative levels of pNF-κBp65 in response to PG/IGF-II were significantly reduced in the presence of curcumin. Differences in relative inhibitory potency of curcumin against IGF-II- vs. PG-stimulated activation of NF-κB was only 20–30%; this difference, in itself, probably does not account for the much larger difference in protective potency of IGF-II vs. PG against antiproliferative and antiapoptotic effects of curcumin (Figs. 1F and 2D).

Unlike unitargeted or multitargeted drugs, dietary agents such as curcumin have been reported to downregulate activation of several signaling pathways and a large array of unrelated membrane proteins (12, 19). Besides NF-κB, pathways known to be targeted directly or indirectly by curcumin, include ERKs, p38MAPK, and c-Src. An important role of p38MAPK activity was demonstrated in experiments with cisplatin-resistant human ovarian carcinoma cells, wherein curcumin was reported to induce apoptosis of resistant cells by significantly reducing levels of p38MAPK activity (48). Radiosensitivity of cancer cells was significantly reduced by either inhibiting p38MAPK or IGF-I-R (6), further suggesting an important role of p38 in mediating biological responses to both PG (32, 44) and ionizing radiation or IGFs (6). Curcumin significantly inhibits pp60c-Src tyrosine kinase (discussed in Ref. 19). Both PG and IGF-II significantly upregulated activation of p44/42 ERKs, p38MAPK, and c-Src (Figs. 5A and 6, A–C). An interesting finding was that curcumin was not very potent in reversing activation of ERKs/c-Src in response to PG/IGF-II. On the other hand, curcumin was extremely potent in inhibiting activation of p38MAPK in response to PG, but not IGF-II (Figs. 5A and 6C). These findings were unexpected. As described above, γ-irradiation of non-small lung cancer cells activates IGF-I-R, resulting in complex formation with p38MAPK and signals for DNA damage response, independent of PI3K (6). Although in the present study, cells were not irradiated, it is possible that ligand-stimulated IGF-I-R also forms a complex with p38MAPK, which is perhaps resistant to proapoptotic effects of curcumin; this intriguing possibility needs to be examined in future studies.

To confirm a possible critical role p38MAPK in mediating resistance of IGF-II-stimulated cells to inhibitory effects of curcumin, activation of p38 was pharmacologically inhibited by a specific inhibitor of p38MAPK. IEC-18 cells, inhibited for p38MAPK activation, were resensitized to proapoptotic effects of curcumin, in the presence of IGF-II (Fig. 6D). Results with IEC-PG clones, and with day 5 Caco-2 cells, suggest for the first time that cells growing in response to endocrine/autocrine IGFs are likely to be resistant to proapoptotic effects of curcumin and that cells may be resensitized to inhibitory effects of curcumin by either inhibiting the activation of p38MAPK or by negating autocrine IGF-II.

Besides the signaling pathways examined in this study, several other pathways are also known to be directly or indirectly impacted by curcumin, including the EGF receptor pathway. In vitro experiments have shown that short-term treatment with curcumin inhibits EGFR kinase activity and EGF-induced tyrosine phosphorylation of EGFR in A431 cells and depletes the cells of HER2/neu protein (reviewed in Ref. 12). Curcumin is a potent inhibitor of ligand-induced activation of EGF and inhibits phosphorylation of EGFR, which occurs extensively in established cancers (reviewed in Ref. 12). Combined treatment with curcumin + oxaliplatin significantly inhibited growth of colon cancer cell lines, associated with decreased expression and activation of EGFR/HER-2/HER-3 (25). At the same time, PG/gastrin peptides upregulate EGFR (reviewed in Ref. 31); it is possible that some of the growth effects measured in response to PG are indirectly mediated via upregulation of EGFR. Potent inhibitory effects of curcumin on PG-stimulated growth may therefore be additionally mediated via inhibition of the EGFR-mediated pathways. The latter possibility may further explain the significant difference in the inhibitory potency of curcumin against PG vs. IGF-II-stimulated growth of cells.

Curcumin may potentially regulate action of membrane proteins by changing physical properties of the membranes (1). Curcumin physically interacts with several proteins including c-Src (reviewed in Refs. 12, 19). In preliminary studies we measured significant inhibition of PG binding with its cognate receptor, annexin2; however, curcumin did not inhibit binding of IGFs to IGF-I-R. The possibility that curcumin may inhibit interaction of PG with annexin2 may also contribute to curcumin-mediated attenuation of elevated pp38MAPK in response to PG (Fig. 6C).

Cancer cells exposed to curcumin for 24 h or longer undergo apoptosis (21). In the present studies we measured significant antiproliferative effects of curcumin on IEC-18 cells, growing in the presence of PG or IGF-II (Figs. 1 and 2). Stark differences were measured in proapoptotic potency of curcumin in the presence of IGF-II vs. PG; cells growing in response to IGF-II were relatively resistant to apoptotic effects of curcumin, whereas cells growing in response to PG were much less resistant (Fig. 2D). Similarly, Gautam et al. (16) reported that curcumin induced inhibition of cell proliferation was not specific to cancer cells and was not always associated with apoptosis. It is possible that resistance to proapoptotic effects
of curcumin may reflect presence of protective growth factors such as IGF-II. In preliminary studies, we had reported that Caco-2 cells, which express high concentrations of IGF-II on days 3–5 of cell culture (36), are almost completely resistant to proapoptotic effects of curcumin at early time points (26); the latter observation with Caco-2 cells was confirmed in the present studies with clones of IEC-18 cells overexpressing IGF-II. Clones expressing hIGF-II were manyfold more resistant to inhibitory effects of curcumin than clones overexpressing PG (Fig. 2A, Table 1). Role of autocrine IGF-II was further confirmed in the background of colon cancer cells; anti-IGF-II antibodies effectively resensitized Caco-2 cells to inhibitory effects of curcumin (Fig. 2B). We have additionally measured significant inhibitory effects of dietary curcumin (2–4%) against development of preneoplastic lesions (aberrant crypt foci) in transgenic mice overexpressing PG in the intestines (Fab-PG mice), to levels measured in wild-type FVB/N mice, in response to azoxymethane (unpublished data from our laboratory). In preliminary studies, we have also examined lengths of isolated colonic crypts (by our published methods; Refs. 44, 45) from mice fed control or 2% dietary curcumin ± 10 nM PG/IGF-II injections, twice daily, for 7 days. Colonic crypt lengths measured in the various groups of mice were in the order of control diet + IGF-II ≈ control-diet + PG ≈ curcumin-diet + IGF-II > * curcumin-diet + PG ≈ curcumin-diet + saline ≈ control-diet + saline. Thus the present in vitro studies with wt IEC-18/IEC-PG vs. IEC-IGF-II/Caco-2 cells and the unpublished in vivo studies (described above) provide strong evidence that growth of intestinal epithelial cells, colonic crypt cells, and colon cancer cells, in the presence of endocrine/autoocrine IGF-II, is likely to be resistant to inhibitory effects of curcumin.

Rationally designed personalized strategies will likely be required for treating diseases such as cancer. Results of the present studies heighten the need to examine inhibitory efficacy of dietary agents, in the presence of physiologically or pathologically relevant endocrine and autocrine growth factors. Our studies strongly suggest that patients positive for high levels of endocrine growth factors, especially IGFs, are likely to be less responsive to inhibitory effects of curcumin; tumors positive for expression of autocrine growth factors, especially IGF-II, can also be extrapolated to be resistant to inhibitory effects of curcumin and may require combinatorial treatments for reducing intracellular p38 and perhaps other signaling molecules.

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DISCLOSURES

None of the authors have any conflict of interest with any entity for any part of these studies.

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