Stimulatory Effects of Insulin-like Growth Factor-I on Growth Plate Chondrogenesis Are Mediated by Nuclear Factor-κB p65*

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Insulin-like growth factor-I (IGF-I) is an important regulator of endochondral ossification. However, little is known about the signaling pathways activated by IGF-I in growth plate chondrocytes. We have previously shown that NF-κB, p65 facilitates growth plate chondrogenesis. In this study, we first cultured rat metatarsal bones with IGF-I and/or pyrrolidine dithiocarbamate (PDTC), a known NF-κB inhibitor. The IGF-I-mediated stimulation of metatarsal growth and growth plate chondrogenesis was neutralized by PDTC. In rat growth plate chondrocytes, IGF-I induced NF-κB-p65 nuclear translocation. The inhibition of NF-κB-p65 expression and activity (by p65 short interfering RNA and PDTC, respectively) in chondrocytes reversed the IGF-I-mediated induction of cell proliferation and differentiation and the IGF-I-mediated prevention of cell apoptosis. Moreover, the inhibition of the phosphatidylinositol 3-kinase and Akt abolished the effects of IGF-I on NF-κB activation. In conclusion, our findings indicate that IGF-I stimulates growth plate chondrogenesis by activating NF-κB-p65 in chondrocytes.

IGF-I2 is a key regulator of longitudinal bone growth, with such role being exerted both during intrauterine and extrauterine life. Knock-out mice for IGF-I exhibit intrauterine growth retardation and experience a subnormal postnatal growth rate (1). A similar growth pattern has been described in a child born with a homozygous IGF-I deletion (2).

The fact that IGF-I null mice have a reduced growth plate height clearly suggests a facilitatory role for IGF-I on growth plate chondrogenesis and, in turn, on longitudinal bone growth. The presence of the IGF-I receptor in growth plate chondrocytes also suggests that IGF-I facilitates longitudinal bone growth directly at the growth plate (3, 4). However, little is known about the specific molecular mechanisms responsible for the IGF-I-mediated induction of growth plate chondrogenesis.

Mammalian NF-κB is a group of transcription factors, including seven members, p65 (RelA), c-Rel, RelB, p50/p105 (NF-κB1), and p52/p100 (NF-κB2) (5). Upon activation by a wide variety of stimuli (proinflammatory cytokines, growth factors, and viral proteins), NF-κB translocates to the nucleus, where it modulates the expression of target genes involved in cell growth, survival, adhesion, and death (6, 7). These target genes include anti-apoptotic (8) as well as pro-apoptotic ones (9), suggesting that the effects of NF-κB on cell growth and survival may depend on the cell type and on the nature of the extracellular stimuli.

Previous evidence indicates that NF-κB exerts a regulatory role in bone growth and development. Mice deficient in both the NF-κB subunits p50 and p52 have retarded growth and shortened long bones (10), suggesting that NF-κB may be involved in bone formation and growth. In addition, we have recently shown that the NF-κB subunit p65 has a facilitatory role on growth plate chondrogenesis (11). Because experimental evidence in a number of cell types suggests a functional interaction between IGF-I and NF-κB (12–18), we hypothesized that IGF-I regulates growth plate chondrogenesis and longitudinal bone growth by inducing the activity of NF-κB in growth plate chondrocytes.

To test our hypothesis, we first cultured whole rat metatarsal bones in the presence of IGF-I and pyrrolidine dithiocarbamate (PDTC, a known NF-κB inhibitor) to study their effects on metatarsal longitudinal growth and growth plate chondrogenesis. Second, we analyzed the effects of IGF-I on the nuclear translocation of NF-κB in growth plate chondrocytes, and the effects of IGF-I, PDTC, and NF-κB p65 siRNA on cultured growth plate chondrocyte proliferation, differentiation, and apoptosis. Finally, we evaluated the effects of the selective inhibition of the IGF-I receptor-activated intracellular signaling pathways on the IGF-I-mediated induction of NF-κB activity.

MATERIALS AND METHODS

Whole Metatarsal Culture—The second, third, and fourth metatarsal bone rudiments were isolated from Sprague-Dawley rat fetuses at 20 days postconception and cultured individually in 24-well plates (19, 20). Each well contained 0.5 ml of minimum essential medium (Invitrogen), supplemented with 0.05 mg/ml ascorbic acid (Sigma), 1 mM sodium glycerophosphate (Sigma), 0.2% bovine serum albumin (Sigma), 100 units/ml...
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penicillin, and 100 μg/ml streptomycin (Invitrogen). Bone rudiments were cultured for 3 days in a humidified incubator with 5% CO₂ in air at 37 °C. The medium was changed on day 2. During the 3-day culture period, metatarsals were cultured in the absence or presence of 100 ng/ml IGF-I, with or without 1 μM pyrrolidone dithiocarbamate (PDTC) (Sigma), a specific NF-κB inhibitor. Animal care was in accordance with the Guide for the Care and Use of Laboratory Animals (DHEW Publication (NIH) 85-23, revised 1996).

Chondrocyte Culture—Metatarsal rudiments isolated from Sprague-Dawley rat fetuses at 20 days postconception were rinsed in PBS, incubated in 0.2% trypsin for 1 h, and then 0.2% collagenase for 3 h. Cell suspension was aspirated repeatedly and filtered through a 70-μm cell strainer, rinsed first in PBS and then in serum-free DMEM, and counted. Chondrocytes were seeded in 100-mm dishes at a density of 5 × 10⁴/cm² in DMEM with 100 units/ml penicillin and 100 μg/ml streptomycin, 50 μg/ml ascorbic acid, and 10% fetal bovine serum. The culture medium was changed at 72-h intervals. Once 70–80% confluence was reached, cells were washed with serum-free medium and treated with 100 ng/ml IGF-I and/or 1 μM PDTC and/or graded concentrations of selective protein kinase inhibitors (EMD Chemicals, Gibbstown, NJ).

Measurement of Metatarsal Longitudinal Growth—The length of each bone rudiment was measured under a dissecting microscope, using an eyepiece micrometer. The eyepiece micrometer was calibrated every day by using a 1-mm stage micrometer. To calculate the metatarsal growth rate, bone length was measured at the beginning and at the end of the 3-day culture period using an eyepiece micrometer in a dissecting microscope. For each treatment group, 48–53 metatarsal bones isolated from 15 to 18 rat fetuses were used. Results represent the mean ± S.E. of three separate experiments.

Quantitative Histology—At the end of the culture period, metatarsals were fixed in 4% phosphate-buffered paraformaldehyde overnight. After routine processing, 3 longitudinal, 5–7-μm-thick sections were obtained from each metatarsal bone and stained with toluidine blue. From each of the three sections, we measured the height of the epiphyseal zone, proliferative zone, and the hypertrophic zone, and we calculated the average value. In the metatarsal growth plate, the epiphyseal zone is characterized by small and rounded cells, irregularly arranged in the cartilage matrix. The proliferative zone includes cells with a flattened shape, arranged in columns parallel to the longitudinal axis of the bone. In the hypertrophic zone, large cells (defined by a height ≥9 μm) form a layer adjacent to the calcified region of the metatarsal bone, the primary ossification center. For each treatment group, 7–9 metatarsal bones (=21–27 sections/group) isolated from 3 to 4 rat fetuses were used. Results represent the mean ± S.E. of three separate experiments. All quantitative histology was performed by a single observer blinded to the treatment category.

[^3]H] Thymidine Incorporation—To assess cell proliferation in the metatarsal growth plate, we measured [^3]H] thymidine incorporation (19). After 3 days of culture, [^3]H] thymidine was added to the culture medium of the rat metatarsal bones at a concentration of 5 μCi/ml (25 Ci/mmol; Amersham Biosciences). Bone rudiments were incubated for an additional 5 h. At the end of the incubation, all bones were fixed in 4% phosphate-buffered paraformaldehyde, embedded in paraffin, and cut in 5–7-μm-thick longitudinal sections. Autoradiography was performed by dipping the slides in Hypercoat emulsion, exposing them for 4 weeks, and then developing them with a Kodak-D19 developer. Sections were counterstained with hematoxylin. For each treatment group, five metatarsal bones isolated from three rat fetuses were sampled, and three sections of each bone (with each section containing both metatarsal growth plates) were analyzed (=15 sections/group). The labeling index was calculated as the number of [^3]H] thymidine-labeled cells per grid divided by the total number of cells per grid. The grid circumscribed a portion of the growth plate zone as viewed through a ×40 objective and generally contained an average of 50 cells. In each bone section, the labeling index was calculated in three distinct grid locations and then averaged separately in the epiphyseal and in the proliferative zones. All determinations were made by the same observer blinded to the treatment category.

To assess proliferation in cultured chondrocytes, 2.5 μCi/well of [^3]H] thymidine (Amersham Biosciences) was added to the culture medium for an additional 3 h at the end of the culture period. Cells were then washed, precipitated with trichloroacetic acid, and lysed in 0.5 M NaOH, 0.5% SDS. Incorporation of [^3]H] thymidine was measured by liquid scintillation counting and normalized by protein content.

RT-PCR—At the end of the culture period, total RNA was extracted from the growth plates of whole rat metatarsal bones or from cultured chondrocytes using the Qiagen RNeasy mini kit (Qiagen Inc., Valencia, CA). The following specific primers were used: rat collagen X (5′ primer, 5′-ATATCTTGGAATCCAGTCC-3′; 3′ primer, 5′-tggg-taccccttagtacg-3′; product size 241 bp) (AJ131848), and rat NF-κB p65 (Santa Cruz Biotechnology, Santa Cruz CA, catalog number sc-61876-PR). The housekeeping gene β-actin (5′ primer, 5′-CTGACAAGACTACCCTCATAGAAATCC-3′; 3′ primer, 5′-CATAGAGGTCTTTACGGTGTCAC-3′; product size 330 bp) (NM_031144) was used as normalization control. The recovered RNA was further processed using First Strand cDNA synthesis kit for RT-PCR (avian myeloblastosis virus) (Roche Diagnostics) to produce cDNA. One microgram of total RNA and 1.6 μg of p(dT)₁₅ primer were incubated for 10 min at 25 °C, followed by incubation for 60 min at 42 °C in the presence of 20 units of avian myeloblastosis virus reverse transcriptase and 50 units of RNase inhibitor in a total 20-μl reaction. The cDNA products were directly used for PCR or stored at −80 °C for later analysis. The reaction (100 μl total volume) was performed using a GeneAmp PCR System 9600 (PerkinElmer Life Sciences) in the presence of 20 pmol of primers, 20 nmol of dNTP, 150 nmol of MgCl₂, 1 × PCR buffer (Expand High Fidelity PCR buffer; Roche Applied Science), and 2.5 units Expand High Fidelity DNA polymerase (Roche Applied Science). The conditions for amplification were 2 min and 30 s at 96 °C, followed by 35 cycles of denaturation for 45 s at 96 °C, annealing for 1 min at 55 °C, elongation for 1 min 30 s at 72 °C, and finally extension for 10 min at 72 °C. PCR products were separated by electrophoresis in a 2% agarose gel with ethidium bromide (1.5 μg/ml).
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TABLE 1
Effects of IGF-I and PDTC on metatarsal longitudinal growth and growth plate chondrogenesis

The following abbreviations are used: EZ, epiphyseal zone; PZ, proliferative zone; HZ, hypertrophic zone.

| Cumulative growth (µm) | Zone height (µm) | Labeling index (%) |
|------------------------|-----------------|--------------------|
| (48–53 bones/group)    | (21–27 sections/group) | (15 sections/group) |
| Control                | EZ              | PZ                | EZ | PZ |
| 264.6 ± 14.9           | 607.1 ± 10.4    | 118.5 ± 7.0       | 85.1 ± 6.1      | 15.6 ± 1.1 | 8.1 ± 0.4 |
| IGF-I                  | 363.5 ± 18.0a   | 696.4 ± 30.2b     | 151.5 ± 7.8b    | 103.8 ± 2.8b | 23.9 ± 0.7a | 13.4 ± 1.2a |
| PDTC                   | 233.3 ± 14.4    | 603.9 ± 8.6       | 120.0 ± 4.1     | 89.0 ± 1.3    | 15.9 ± 2.7  | 7.8 ± 1.7   |
| IGF-I + PDTC           | 286.9 ± 15.6c   | 694.6 ± 46.5c     | 120.2 ± 6.3c    | 85.3 ± 3.6c   | 17.7 ± 1.5c | 8.8 ± 1.5c  |

*p = <0.01 versus control.

*p = <0.05 versus control.

*p = <0.01 versus IGF-I.

*p = <0.05 versus IGF-I.

p65 siRNA Transfection—Chondrocytes were transfected with an siRNA targeted for rat NF-κB p65 (Santa Cruz Biotechnology, catalog number sc-61876) using Lipofectamine 2000 (Invitrogen). An siRNA consisting of a scrambled sequence of NF-κB p65 was similarly transfected as control siRNA. One day before transfection, cells were plated in 500 µl of growth medium without antibiotics such that they were 30–50% confluent at the time of transfection. The transfected cells were cultured in DMEM containing 10% fetal calf serum for 72 h after transfection.

To determine transfection efficiency, we transfected chondrocytes with fluorescein isothiocyanate-conjugated control siRNA (Santa Cruz Biotechnology, catalog number sc-36869) in two separate experiments. Transfection efficiency was 45.0 ± 2.4% (range of 30.8–51.4%). To determine whether p65 siRNA specifically silenced NF-κB p65 expression, we evaluated NF-κB p65 mRNA and protein expression in transfected chondrocytes by RT-PCR and Western blot, respectively.

In Situ Cell Death—Chondrocytes were treated for 24 h with 1 mM sodium nitroprusside (SNP, Sigma), a known inducer of apoptosis (21, 22), in the presence of 100 ng/ml IGF-I and/or 1 µM PDTC. At the end of the culture period, cultured chondrocytes were washed with PBS three times and fixed in −10°C methanol for 5 min, and then air-dried. Apoptotic cells were identified by terminal deoxynucleotidyltransferase-mediated deoxyuridine triphosphate nick end labeling, according to the manufacturer’s instructions (TdT-FragEL kit; Oncogene Research Products, Boston). A positive control was generated by adding 1 µg/µl DNase I in 1× TBS, 1 mM MgSO4 following the NF-κB response element was immobilized onto the bottom of wells of a 96-well plate. NF-κB p65 contained in the nuclear extract was detected by addition of a specific primary antibody directed against NF-κB p65. A secondary antibody conjugated to horseradish peroxidase was added to provide a sensitive colorimetric readout at 450 nm. Nuclei were extracted from chondrocytes treated for 24 h with 100 ng/ml IGF-I, and/or 1 µM PDTC, and/or the following specific protein kinase inhibitors (23) wortmannin (PI3K inhibitor); U0126 (MAPK inhibitor); Akti 1/2 (Akt inhibitor); bisindolylmaleimide I (PKC inhibitor); PDTC, and/or the following specific protein kinase inhibitors (23) wortmannin (PI3K inhibitor); U0126 (MAPK inhibitor); Akti 1/2 (Akt inhibitor); bisindolylmaleimide I (PKC inhibitor); H-89 (PKA inhibitor). All these inhibitors were purchased from EMD Chemicals, Gibbstown, NJ. Data are expressed as the mean ± S.E. of optical density per µg of protein and represent three separate experiments.

Statistics—All data are expressed as the mean ± S.E. Statistical significance was determined by t test or by analysis of variance.

RESULTS

Effects of IGF-I and PDTC on Metatarsal Longitudinal Growth and Growth Plate Chondrogenesis—During the 3 days of the culture period, 100 ng/ml IGF-I significantly stimulated metatarsal longitudinal growth (n = 48–53 bones/group; Table 1), whereas co-treatment of IGF-I and 1 µM PDTC reversed this stimulatory effect. Because the rate of longitudinal bone growth depends primarily on the rate of growth plate chondrogenesis, we evaluated the effects of IGF-I on chondrocyte proliferation and chondrocyte hypertrophy/differentiation. To determine the site of the growth plate in which chondrocyte proliferation occurred, we examined the in situ [3H]thymidine incorporation into the metatarsal rudiments at the end of the culture period. IGF-I significantly increased [3H]thymidine incorporation into the growth plate epiphyseal and proliferative zones (represent-
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FIGURE 1. Effects of IGF-I and PDTC on in situ [3H]thymidine incorporation and collagen X mRNA expression in the metatarsal growth plate. Fetal rat metatarsals (20 days post-coitum) were cultured for 3 days in serum-free minimum essential medium in the absence (A) or presence of 100 ng/ml IGF-I (B), 1 μM PDTC (C), and a combination of IGF-I and PDTC (D). After 3 days in culture, [3H]thymidine was added to the culture medium at a final concentration of 5 μCi/ml. Bone rudiments were incubated for an additional 3 h and subsequently processed as described under “Materials and Methods.” Metatarsal longitudinal sections were counterstained with hematoxylin. A representative [3H]thymidine-labeled cell is indicated by the arrow. EZ, epiphyseal zone; PZ, proliferative zone. E, RT-PCR analysis of collagen X mRNA expression in metatarsal bones. Total RNA was extracted from the growth plates of metatarsals treated with or without IGF-1 (100 ng/ml) in the presence or absence of 1 μM PDTC and then reverse-transcribed to cDNA (n = 15 metatarsals/group). The housekeeping gene β-actin was used as normalization control. PCR products were separated by electrophoresis in a 2% agarose gel with ethidium bromide. Results are representative of three separate experiments.

FIGURE 2. Effects of IGF-I, PDTC, and p65 siRNA on chondrocyte proliferation and differentiation. A and B, chondrocytes isolated from fetal rat metatarsal growth plates were washed with fresh serum-free DMEM, seeded in 24-well plate, and transfected with p65 siRNA or a control siRNA for 72 h. After transfection, NF-κB p65 mRNA (A) and protein (B) expression was analyzed by RT-PCR and Western blot, respectively. C, transfected chondrocytes were then cultured without or with 100 ng/ml IGF-I and/or 1 μM PDTC. At the end of culture period, chondrocytes were added with 2.5 μCi/well of [3H]thymidine (Amersham Biosciences) to the culture medium for an additional 3 h. Cells were then washed, precipitated with trichloroacetic acid, and lysed in 0.5 M NaOH, 0.5% SDS. Incorporation of [3H]thymidine was measured by liquid scintillation counting and normalized by protein content. Results were expressed as cpm/mg protein. D, total RNA was extracted from chondrocytes transfected with p65 siRNA or control siRNA, and cultured without or with 100 ng/ml IGF-I and/or 1 μM PDTC. RNA was then reverse-transcribed to cDNA. The housekeeping gene β-actin was used as normalization control. PCR products were separated by electrophoresis in a 2% agarose gel with ethidium bromide. Results are representative of three separate experiments.

Effects of IGF-I, PDTC, and p65 siRNA on Chondrocyte Proliferation, Differentiation, and Apoptosis—
To determine whether IGF-I functionally interacts with NF-κB p65 in chondrocytes, we first transfected chondrocytes isolated from fetal rat metatarsal growth plates with a rat NF-κB p65 siRNA or a control siRNA. p65 siRNA-transfected chondrocytes exhibited reduced p65 mRNA and protein expression (Fig. 2, A and B, respectively) compared with control siRNA-transfected chondrocytes.

To confirm the findings observed in the whole metatarsal bones, we evaluated the effects of IGF-I on transfected growth plate chondrocyte proliferation, differentiation, and apoptosis. In chondrocytes isolated from metatarsal growth plates and transfected with control siRNA, 100 ng/ml IGF-I induced chondrocyte proliferation (assessed by [3H]thymidine incorporation; IGF-I versus control, Fig. 2C) as well as dif-
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Effects of PI3K and Akt inhibitors on the IGF-I-mediated induction of NF-κB-DNA binding activity—To determine whether IGF-I specifically induces NF-κB p65 activation, we evaluated the NF-κB-p65 DNA binding activity by using an NF-κB p65 transcription factor enzyme-linked immunosorbent assay. IGF-I significantly increased the NF-κB p65-DNA binding activity in control-siRNA-transfected cells (Table 2), whereas co-treatment of 1 μM PDTC and IGF-I reversed such stimulatory effect of IGF-I (Table 2). PDTC alone did not modify the NF-κB p65-DNA binding activity. As shown by us previously (11), p65 siRNA-transfected cells exhibited decreased NF-κB p65-DNA binding activity when compared with control siRNA-transfected cells (Table 2). However, the addition of 100 ng/ml IGF-I to the culture medium fully normalized the NF-κB p65-DNA binding activity (Table 2).

To determine which intracellular signaling pathway(s) mediates the effects of IGF-I on NF-κB p65 activity, we cultured chondrocytes in the presence of 100 ng/ml IGF-I, with or without specific inhibitors of each of the signaling pathways activated by IGF-I. The addition of 10 μM wortmannin (a PI3K inhibitor) or 10 μM Akti 1/2 (a Akt inhibitor) to the culture medium of IGF-I-treated chondrocytes significantly reversed the stimulatory effects of IGF-I on NF-κB p65 DNA binding activity (Table 3 and Fig. 4, A and B). In contrast, the addition of U0126 (a MAPK inhibitor), H-89 (a PKA inhibitor), or bisindolylmaleimide I (a PKC inhibitor) did not modify the IGF-I-mediated increase of NF-κB p65 activity (Fig. 4, C–E). In the absence of IGF-I in the culture medium, none of the above

TABLE 2
Effects of IGF-I, PDTC, and p65 siRNA on NF-κB-DNA binding activity

| Treatment             | A_{450/μg protein} |
|-----------------------|--------------------|
| Control siRNA         | 0.098 ± 0.007      |
| IGF-I + control siRNA | 0.248 ± 0.025     |
| PDTC + control siRNA  | 0.111 ± 0.009     |
| p65 siRNA             | 0.061 ± 0.001     |
| IGF-I + PDTC + control siRNA | 0.120 ± 0.007     |
| IGF-I + p65 siRNA     | 0.111 ± 0.005     |

* p was <0.05 versus control.
† p was <0.05 versus IGF-I.

TABLE 3
Effects of PI3K and Akt inhibitors on the IGF-I-mediated induction of NF-κB-DNA binding activity

| Treatment              | A_{450/μg protein} |
|------------------------|--------------------|
| Control                | 0.09  ± 0.006     |
| IGF-I                  | 0.273 ± 0.009*    |
| IGF-I + 10 μM wortmannin | 0.130 ± 0.01*     |
| IGF-I + 10 μM Akti 1/2 | 0.137 ± 0.002*    |

* p was <0.05 versus control.
† p was <0.05 versus IGF-I.

ferentiation (assessed by collagen X mRNA expression, Fig. 2D). The co-treatment of IGF-I with 1 μM PDTC reversed these stimulatory effects (IGF-I + PDTC versus IGF-I, Fig. 2, C and D). The transfection of cultured chondrocytes with NF-κB p65 siRNA reduced [³H]thymidine incorporation compared with control siRNA-transfected chondrocytes (p65 siRNA versus control, Fig. 2C), with such effect being reversed by 100 ng/ml IGF-I (IGF-I + p65 siRNA versus p65 siRNA, Fig. 2C). The addition of IGF-I in the culture medium of p65 siRNA-transfected chondrocytes did not modify collagen X expression (IGF-I + p65 siRNA versus p65 siRNA, Fig. 2D).

In light of the regulatory role of IGF-I and NF-κB on apoptosis in other cell types, we evaluated the effects of IGF-I, PDTC, and p65 siRNA on chondrocyte apoptosis by assessing in situ cell death and caspase-3 activity. Chondrocytes cultured with 1 mM SNP exhibited increased cell death (representative photographs, Fig. 3B, and apoptotic index, Fig. 3G) and caspase-3 activity (Fig. 3H) when compared with control chondrocytes. The addition of 100 ng/ml IGF-I to the culture medium of the SNP-treated chondrocytes prevented the SNP-mediated increase of cell death (representative photograph, Fig. 3C, and apoptotic index, Fig. 3G) and caspase-3 activity (Fig. 3H). However, co-treatment of 1 μM PDTC + 100 ng/ml IGF-I reversed the antiapoptotic effects of IGF-I on the SNP-induced cell death and caspase-3 activity (representative photograph, Fig. 3D; apoptotic index, Fig. 3G, and caspase-3 activity, Fig. 3H).

FIGURE 3. Effects of IGF-I and PDTC on chondrocyte in situ cell death and caspase-3 activity. Cultured chondrocytes were cultured without or with SNP (1 mM), IGF-I (100 ng/ml), and/or PTDC (1 μM). A–F, at the end of the culture period, cultured chondrocytes were washed with PBS three times, fixed in −10 °C methanol for 5 min, and then air-dried. Apoptotic cells were identified by terminal deoxynucleotidyltransferase-mediated deoxyuridine triphosphate nick end labeling assay, according to the manufacturer’s instructions. A representative apoptotic cell is indicated by the arrow. A, control chondrocytes. B, chondrocytes treated with SNP. C, chondrocytes treated with SNP and IGF-I. D, chondrocytes treated with IGF-I and PTDC. E, chondrocytes treated with PTDC. F, chondrocytes treated with IGF-I. G, the apoptotic index was calculated as described under ‘Materials and Methods.’ H, at the end of the culture period, cytosolic caspase-3 activity was analyzed by a colorimetric assay. Results represent the mean ± S.E. of three separate experiments.

Effects of IGF-I on NF-κB p65-DNA Binding—To determine whether IGF-I specifically induces NF-κB p65 activation, we evaluated the NF-κB-DNA binding activity by using an NF-κB p65 transcription factor enzyme-linked immunosorbent assay. IGF-I significantly increased the NF-κB p65-DNA binding activity in control-siRNA-transfected cells (Table 2), whereas co-treatment of 1 μM PDTC and IGF-I reversed such stimulatory effect of IGF-I (Table 2). PDTC alone did not modify the NF-κB p65-DNA binding activity. As shown by us previously (11), p65 siRNA-transfected cells exhibited decreased NF-κB p65-DNA binding activity when compared with control siRNA-transfected cells (Table 2). However, the addition of 100 ng/ml IGF-I to the culture medium fully normalized the NF-κB p65-DNA binding activity (Table 2).
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**FIGURE 4. Effects of protein kinase inhibitors on the IGF-I-mediated induction of NF-κB p65-DNA binding activity.** Chondrocytes were cultured in the presence or absence of IGF-I (100 ng/ml), with or without the specific inhibitors of each of the IGF-dependent signaling pathways (1–10 μM). NF-κB p65-DNA binding activity was determined by an enzyme-linked immunosorbent assay, according to the manufacturer’s instructions. A, wortmannin (PI3K inhibitor); B, Akti 1/2 (Akt inhibitor); C, U0126 (MAPK inhibitor); D, H89 (PKA inhibitor); E, bisindolylmaleimide I (BIS) (PKC inhibitor).

Inhibitors had any effect on the NF-κB p65-DNA binding activity in chondrocytes (Fig. 4).

**DISCUSSION**

In mammals, the rate of longitudinal bone growth depends primarily on the rate of growth plate chondrogenesis. Growth plate chondrocyte proliferation, hypertrophy/differentiation, and extracellular matrix secretion lead to the formation of new cartilage, chondrogenesis (24). Simultaneously, while the terminally differentiated chondrocytes die by apoptosis, the growth plate is invaded from the metaphysis by blood vessels and bone cell precursors, which remodel the cartilage into bone tissue (25). The net result of these two well coordinated processes, chondrogenesis and ossification, is long bone elongation. Growth plate chondrogenesis is regulated by a network of endocrine and paracrine factors, which modulate the chondrocyte function via several intracellular transcription factors.

Our study demonstrates that IGF-I stimulates metatarsal longitudinal growth. Such an effect results from the stimulation of the two main cellular events of chondrogenesis: growth plate chondrocyte proliferation (reflected by the increased percentage of proliferating cells in the epiphysial and proliferative zones, and the increased height of both zones), and chondrocyte differentiation/hypertrophy (increased hypertrophic zone height and induced collagen X mRNA expression). These effects observed in the metatarsal growth plate were confirmed by additional experiments in chondrocytes cultured in the presence of IGF-I. The stimulatory effects of IGF-I on longitudinal bone growth may also be related to its anti-apoptotic effects in the growth plate, as suggested by the IGF-I-mediated prevention of chondrocyte apoptosis induced by sodium nitroprusside.

IGF-I is a critical regulator of skeletal growth. Mice lacking the *igf1* or the *igfr* genes exhibit prenatal growth failure, with their weights being ~60 or 45% of those of wild-type mice, respectively (1). The *igf1* null mice have ~30% of normal weight postnatally at steady state, reflecting a progressively decelerating growth rate (1). Their tibial growth rate is reduced by ~35% between postnatal days 20 and 40, whereas their growth plate exhibits an expanded epiphysial/resting zone and a significantly reduced hypertrophic zone (26). However, such morphological findings may not be IGF-I-mediated, because *igf1* null mice demonstrate increased growth hormone and IGF-II expression and action. The effects elicited by the systemic administration of IGF-I to hypophysectomized rats suggest that IGF-I stimulates growth plate chondrocytes at all stages of differentiation (27). Local infusion of IGF-I in the rabbit tibial growth plate results in an acceleration of the tibial growth rate and in an increased number of proliferative and hypertrophic chondrocytes in the growth plate (28). Consistent with our findings, it has previously shown that, in cultured fetal rat metatarsal bones, IGF-I stimulates longitudinal bone growth (29), whereas in fetal mouse metatarsals it induces cell proliferation and hypertrophy in the growth plate (30).

IGF-I exerts its biological action by binding and activating the IGF-I receptor (31), a member of the receptor tyrosine kinase family of growth factor receptors. The binding of IGF-I to IGF-I receptor triggers first autophosphorylation of the receptor and then tyrosyl phosphorylation of insulin receptor substrate-1 (IRS-1), which functions as a docking protein for the downstream signal transduction pathways (32, 33). The PI3K, the MAPK/ERK1/2, the PKA, and the PKC intracellular cascades have been shown to mediate the mitogenic, differentiating, and anti-apoptotic effects of IGF-I (34).

IRS-1 null mice exhibit osteopenia, low bone turn over, shorter limbs, and early closure of the growth plates of the long bones (35). Although these findings suggest that IRS-1 signaling is important for skeletal growth, they do not necessarily reflect the physiological role of IGF-I and IRS-1 in chondrocytes.

Previous studies have shown that IGF-I modulates chondrocyte proliferation, differentiation, and apoptosis by activating multiple intracellular signaling pathways. IGF-I stimulates rat growth plate chondrocyte proliferation by activating both the MAPK and the PI3K pathways (36), which induces proliferation of RCJ3.1C5.18 and ATDC5 cells (mesenchymal cells with chondrogenic potential) through the MAPK (37, 38) as well as the PI3K, the PKA, and the PKC pathways (37). Regarding the effects of IGF-I on chondrocyte terminal differentiation and apoptosis, they both appear to be primarily mediated by the PI3K/Akt signaling pathway (36–39). Although this experimental evidence has shed light on the signaling pathways involved, little is known on the transcription factor(s) ultimately mediating the effects of IGF-I on growth plate chondrogenesis.

We have recently demonstrated that NF-κB-p65, expressed in growth plate chondrocytes, facilitates longitudinal bone growth by inducing chondrocyte proliferation and differentiation and by preventing apoptosis (11). Because these effects are
identical to those elicited by IGF-I in the growth plate, we hypothesized that the IGF-I effects on chondrogenesis are mediated by NF-κB-p65. A functional interaction between IGF-I and NF-κB has been shown in some cell types and not in others, and as IGF-I does not affect NF-κB activity in murine pro-B cells (40) and vascular endothelial cells (15), it prevents apoptosis in rat neurons (16) and induces collagen I expression in lung fibroblasts (12) by augmenting NF-κB nuclear translation via the PI3K pathway.

Our findings indicate that IGF-I induces NF-κB-p65 activation in growth plate chondrocytes. More importantly, the inhibition of NF-κB activity by PDTC (a specific NF-κB inhibitor) neutralized the stimulatory effects of IGF-I on metatarsal longitudinal growth and on cell proliferation in the epiphyseal and proliferative zones of the metatarsal growth plate. The lack of a neutralizing effect of PDTC on the IGF-I-mediated increase of the epiphyseal zone height (in contrast to the effect on the proliferative zone height) may depend on the limited contribution of cell proliferation to the thickness of this zone compared with the proliferative zone. In addition, PDTC reversed the stimulatory effects of IGF-I on growth plate chondrocyte differentiation/hypertrophy, as assessed by collagen X mRNA expression in the metatarsal growth plate and by growth plate histology. Our experiments in cultured chondrocytes confirmed the effects of PDTC on the IGF-I-mediated induction of cell proliferation and differentiation in the metatarsal growth plate. Furthermore, the addition of PDTC to the culture medium reversed the anti-apoptotic effects of IGF-I in cultured chondrocytes, as assessed by in situ cell death and caspase-3 activity. Although the selective inhibition of NF-κB-p65 expression in chondrocytes by siRNA led to decreased chondrocyte proliferation, 1 μg PDTC alone did not affect this cellular process. Such difference would suggest a stronger inhibition of the basal (unstimulated) NF-κB-p65 activity caused by p65 siRNA compared with PDTC. The neutralization of the p65 siRNA effects on chondrocyte proliferation by IGF-I further support a functional interaction between NF-κB-p65 and IGF-I in the regulation of growth plate chondrogenesis and longitudinal bone growth. To determine which one(s) of the IGF-I-dependent intracellular signaling pathways mediates the stimulation of NF-κB-p65 activity by IGF-I, we analyzed the effects of the inhibition of each of the main protein kinases involved in the IGF-I-dependent signaling cascades. The selective inhibition of PI3K and Akt prevented the induction of NF-κB-p65 activity by IGF-I in chondrocytes. In contrast, the inhibition of PKA, PKC, or MAPK did not modify such an IGF-I effect. These findings are consistent with previous studies conducted in other cell types. It has been shown that IGF-I activates NF-κB in fetal lung fibroblasts (12), endothelial cells (15), and in multiple myeloma cells (14) through PI3K. In a chondrocytic cell line, the activation of NF-κB by PI3K/Akt was found to be triggered by bone morphogenetic protein-2 (43), another growth factor expressed in the growth plate known to stimulate growth plate chondrogenesis (44). With respect to the molecular mechanisms linking PI3K/Akt and NF-κB, evidence indicates that the activation of NF-κB p65 by the PI3K/Akt pathway involves the p65 Ser-536 phosphorylation by the IκB kinase (41, 42, 45).

In conclusion, our study suggests that IGF-I promotes longitudinal bone growth and all the main cellular events characterizing growth plate chondrogenesis by activating, at least in part, NF-κB-p65 via the PI3K/Akt pathway.

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