Insulin-like growth factor-I (IGF-I) may play an important role in the development of renal hypertrophy. In this study we determined the effect of IGF-I on cultured mesangial cells (MCs) and examined activation of key signaling pathways. IGF-I induced hypertrophy as determined by an increase in cell size and an increase in protein to DNA ratio and increased accumulation of extracellular matrix (ECM) proteins. IGF-I also activated both Erk1/Erk2 MAPK and phosphatidylinositol 3-kinase (PI3K) in MCs. Inhibition of either MAPK or PI3K, however, had no effect on IGF-I-induced hypertrophy or ECM production. Next, we examined the effect of IGF-I on activation of the calcium-dependent phosphatase calcineurin. IGF-I treatment stimulated calcineurin activity and increased the protein levels of calcineurin and the calcineurin binding protein, calmodulin. Cyclosporin A, an inhibitor of calcineurin, blocked both IGF-I-mediated hypertrophy and up-regulation of ECM. In addition, calcineurin resulted in sustained Akt activation, indicating possible cross-talk with other signaling pathways. Finally, IGF-I treatment resulted in the calcineurin-independent nuclear localization of NFATc1. Therefore, IGF-I induces hypertrophy and increases ECM accumulation in MCs. IGF-I-mediated hypertrophy is associated with activation of Erk1/Erk2 MAPK and PI3K but does not require either of these pathways. Instead, IGF-I mediates hypertrophy via a calcineurin-dependent pathway.

In response to stress or injury, kidney tissue undergoes hypertrophy, and to a lesser extent hyperplasia, resulting in a net gain in the size of the kidney. Glomeruli, the filtering microvascular structures, are particularly susceptible to hypertrophy, which eventuates in fibrosis. At the cellular level, hypertrophy is characterized by cessation of the cell cycle at G1, a halt in DNA synthesis, and continued production and/or decreased degradation of cellular proteins. The net result is an increase in protein concentration disproportionate to DNA and an increase in the overall size of the cell. In addition to the increase in cell size, expansion of the extracellular matrix (ECM), including fibronectin and collagen type IV, contributes to tissue hypertrophy. Studies in humans and in animal models of renal hypertrophy indicate that early hypertrophy and ECM accumulation are potentially reversible. Therefore, understanding the mechanisms that are required for the induction and maintenance of hypertrophy and ECM accumulation by growth factors, hormones, and cytokines may be critical for developing therapies that prevent or reverse renal hypertrophy.

The insulin-like growth factor (IGF) system has been implicated in glomerular hypertrophy. In patients with type I diabetes, elevated amounts of IGF-I in the urine are associated with hypertrophy and progression of kidney disease. Moreover, endogenous kidney IGF-I levels are elevated within 2–3 days of streptozotocin-induced type I diabetes in rats (4, 5) and IGF-I receptor is up-regulated after prolonged hyperglycemia (6). Although overexpression of growth hormone (which regulates IGF-I) causes both hypertrophy and sclerosis of mouse kidneys (7), increased circulating levels of IGF-I result primarily in hypertrophy (8). This suggests that endogenous rather than circulating levels of IGF-I mediate hypertrophy and subsequent sclerosis. Alternatively, IGF-I is necessary for hypertrophy but may be insufficient for development of sclerosis. Flyvbjerg et al. (9) found that treatment of diabetic rats with octreotide, a somatostatin inhibitor, decreased circulating IGF-I and IGF-II levels. Moreover, kidney hypertrophy was decreased and urinary albumin excretion was blocked (9). Several other studies support this finding (2, 10). Although IGF-I is undoubtedly an important growth factor that mediates vascular renal pathalogy, very little is known about the signaling pathways activated by IGF-I in renal cells.

In some cells, IGF-I commonly signals through two main pathways, the phosphatidylinositol 3-kinase (PI3K) and Erk1/Erk2 MAPK pathways (11). Both are activated following association of insulin receptor substrate (IRS) proteins with the IGF-I receptor and coupling of the activated tyrosine kinase receptor to downstream signaling targets. However, there is very little evidence regarding what pathway(s) IGF-I activates in specific renal cells, including those derived from the glomerulus. di Mari et al. (12) showed that IGF-I treatment results in activation of Erk1/Erk2 MAPK in proximal tubule cells. No evidence for phosphorylation of the adaptor proteins IRS1 and IRS2 by IGF-I in cultured mesangial cells. Mesangial cells constitute one third of glomerular cell populations and contribute to hypertrophy of glomeruli seen in cell; MAPK, mitogen-activated protein kinase; PI3K, phosphatidylinositol 3-kinase; SfM, serum-free medium; NFAT, nuclear factor of activated T cells; ERK, extracellular signal-regulated kinase; IRS, insulin receptor substrate; PBS, phosphate-buffered saline; FMSF, phenyl-methylsulfonyl fluoride; MBP, myelin basic protein; PAGE, polyacrylamide gel electrophoresis; BSA, bovine serum albumin; MOPS, 4-morpholinepropanesulfonic acid; DTT, dithiothreitol; FACS, fluorescence-activated cell sorting.
Calcineurin Is Required for IGF-I-mediated Hypertrophy

In this study, we examined the effect of IGF-I on cultured MCs from glomeruli of rats and evaluated both proliferative and hypertrophic effects. We also examined the signaling pathways activated by IGF-I, including the MAPK and PI3K pathways. In addition, we investigated IGF-I-mediated activation of other pathways, including the calcium-dependent serine/threonine phosphatase, calcineurin. Accordingly, we determined what effect inhibition of these pathways has on IGF-I-mediated hypertrophy and ECM production. Finally, we demonstrated the significance of calcineurin activation by showing IGF-I-mediated nuclear translocation of the calcineurin substrate, nuclear factor of activated T cells- cl (NFATc1).

EXPERIMENTAL PROCEDURES

Materials

Receptor grade recombinant human IGF-I was purchased from GroPep (Adelaide, Australia), and recombinant human TGFβ1 was obtained from R&D Systems, Inc. (Minneapolis, MN). F98069 was from Calbiochem (La Jolla, CA). Wortmannin, cyclosporin A, calcium ionophore A23187, and anti-calcineurin antibodies were from Transduction Laboratories (San Diego, CA), and anti-NFATc1 antibody was purchased from Chemicon (Temecula, CA), anti-phospho Akt, anti-phospho-Erk1/Erk2, and anti-calcineurin antibodies were from Transduction Laboratories (San Diego, CA), and anti-NFATc1 antibody was from Santa Cruz Biotechnology (Santa Cruz, CA).

Cell Culture

Rat MCs were cultured from glomeruli isolated by differential sieving as previously described (14). Epithelial cells were removed by digestion with collagenase, and glomerular cores were cultured in RPMI (Life Technologies, Inc., Gaithersburg, MD) supplemented with antibiotics and 17% fetal calf serum. For these experiments, rat MCs that have been maintained in our laboratory (University of Texas Health Science Center, San Antonio, TX) were used between passage 26 and 32.

Hypertrophy

FACS—MCs were plated in 60-mm plates and allowed to grow to 80–90% confluence. Medium was then changed to serum-free media (SFM) for 24 h, and the cells were treated as indicated. After treatment, MCs were harvested by trypsinization, washed with 1× PBS, centrifuged at 5000 rpm for 2 min, and then resuspended in ice-cold 70% ethanol added dropwise while vortexing. Ethanol-fixed MCs were then analyzed by forward light scattering on a Becton Dickinson flow cytometer.

Protein/DNA Ratio—MCs were plated in triplicate in 12-well plates and allowed to grow to 80–90% confluence. Medium was changed to SFM for 24 h, and the cells were treated as indicated. Each well was washed, collected by trypsinization, and split into two equal aliquots. Both samples were washed with 1× PBS and centrifuged at 5000 rpm for 2 min. One aliquot was resuspended in TNESV protein lysis buffer (50 mM Tris-HCl, pH 7.4, 2 mM EDTA, 1% Nonidet P-40, 100 mM NaCl, 10 mM sodium orthovanadate, 100 μg/ml leupeptin, 20 μg/ml apro tin, and 10−7 M phenylmethanesulfonyl (PMSF)) and the other in ice-cold 70% ethanol added dropwise while vortexing. Ethanol-fixed MCs were then analyzed by forward light scattering on a Becton Dickinson flow cytometer.

Kinase Assay

MCs were plated in 60-mm plates and allowed to grow to 80–90% confluence (~2 days). Medium was then changed to SFM for 24 h, and the cells were treated as indicated. Erk1/Erk2 MAPK—Erk1/Erk2 was immunoprecipitated from 100 μg of total protein using anti-Erk1 antibody (Santa Cruz Biotechnology) followed by protein A-Sepharose beads. Immunocomplexes were washed three times in TNESV buffer and resuspended in kinase buffer (10 mM HEPES, pH 7.4, 10 mM MgCl2, 0.5 mM dithiothreitol, and 0.5 mM Na3VO4) in the presence of 0.5 mg/ml myelin basic protein (MBP), 25 μM cold ATP, and 1 μCi of [γ-32P]ATP. Reactions were incubated for 30 min at 30°C followed by 10-min incubation on ice. Phosphorylated MBF was resolved on 12% SDS-PAGE, the gel was dried, and an exposure was made onto film.
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**Fig. 2.** IGF-I treatment increases expression of collagen type IV and fibronectin proteins. Near-confluent cells were serum-starved for 24 h and then treated with SFM alone (control), IGF-I (100 ng/ml), TGFβ (1 ng/ml), or IGF-I + TGFβ for 72 h. A, total cellular proteins were collected and separated by SDS-PAGE. Collagen type IV and fibronectin were detected by indirect immunoblotting. B, cells were fixed in methanol, and fibronectin and collagen type IV were visualized using anti-fibronectin antibody followed by a Texas Red-conjugated anti-goat secondary antibody (e–h), a and e, SFM (control); b and f, IGF-I; c and g, TGFβ; d and h, IGF-I plus TGFβ.

**PI3K—Kinase activity was determined as described previously (16).** Briefly, tyrosine-phosphorylated proteins were immunoprecipitated from 100 μg of total sample by incubation with anti-phosphotyrosine antibody for 30 min on ice followed by addition of protein A-Sepharose beads and additional incubation on ice for 2 h. Immunocomplexes were washed three times with lysis buffer, once with PBS, once with buffer A (0.5 mM LiCl, 0.1 M Tris-HCl, pH 7.5, 1 mM Na3VO4), once with double-distilled water, and once with buffer B (0.1 M NaCl, 0.5 mM EDTA, 20 mM Tris-HCl, pH 7.5). The immunocomplexes were then resuspended in 50 μl of PI3K buffer (20 mM Tris-HCl, pH 7.5, 0.1 mM NaCl, and 0.5 mM EGTA). 0.5 μg of 20 mg/ml PI was added and incubated at 25 °C for 10 min. A mixture of 1 μl of 1 M MgCl2 and 10 μCi of [γ-32P]ATP was added, and the mixture was incubated at room temperature for 10 min. 150 μl of a mixture of chloroform, methanol, and 11.6× M HCl (50:100:1, v/v/v) was added to stop the reaction, and an additional 100 μl of chloroform was added. The organic layer was extracted and washed with methanol and 1× HCl (1:1). The reaction was dried overnight and resuspended in 10 μl of chloroform. The samples were separated by thin-layer chromatography and developed with CHCl3/MeOH/28% NH4OH/H2O (129:114:15:21). The spots were visualized by autoradiography.

**Western Blots**

MCs were plated in 60-mm dishes and allowed to grow to 80–90% confluency (~2 days). Medium was then changed to SFM for 24 h, and the cells were treated as indicated. Following treatment, the cells were fixed and permeabilized in 100% methanol for 5 min and then rehydrated in PBS-0.1% BSA for 15 min. Cells were then blocked with the appropriate IgG for 15 min and then primary antibodies were added for 30 min. Anti-fibronectin, anti-collagen type IV, and anti-NFATc1 were all added at a concentration of 15 μg/ml. Cells were washed three times for 5 min with PBS-0.1% BSA before addition of secondary antibodies as indicated. All secondary antibodies were used at a dilution of 1–100. Finally, cells were washed three times for 5 min with PBS-0.1% BSA, the chambers were removed from the slides, and the coverslips were mounted with Crystal Mount (Biomeda, Foster City, CA). Cells were viewed by fluorescence microscopy.

**Calcineurin Phosphatase Assay**

Calcineurin phosphatase activity was determined following a protocol published by Fruman et al. (17). Briefly, the RII peptide was phosphorylated in vitro with 250 units of recombinant PKA, 50 μM ATP, 50 μCi of [γ-32P]ATP, 0.15 μM RII, and 500 μM of 2× reaction buffer (40 mM MOPS, 4 mM MgCl2, 0.1 mM CaCl2, 0.4 mM EDTA, 0.8 mM EGTA, 0.5 mM DTT, 0.1 mg/ml BSA). Lysates were prepared by resuspending cells in a hypotonic lysis buffer (50 mM Tris, pH 7.5, 1 mM EDTA, 1 mM EGTA, 0.5 mM DTT, 50 μg/ml PMSF, 10 μg/ml leupeptin, 10 μg/ml aprotinin) followed by 3 cycles of freeze thawing in liquid nitrogen and a 30 °C water bath. Calcineurin activity in each sample was determined by incubating equal parts lystate, 3× reaction buffer (40 mM Tris, pH 7.5, 0.1 mM NaCl, 6 mM MgCl2, 0.1 mM CaCl2, 0.5 mM DTT, 500 mM okadaic acid, and 0.1 mg/ml BSA), and labeled RII peptide at 30 °C for 10 min. The reaction was stopped by addition of 0.1 M KPO4 in 5% trichloroacetic acid. To determine the amount of phosphate released by calcineurin in each sample, reactions were then added to PolyPrep columns (Bio-Rad, Hercules, CA) containing AG-50X Dowex ion exchange resin (Bio-Rad) prepared as described in a previous study (17). Finally, 5 ml of scintillation fluid was added to the flow-through from each column, and the released phosphate was measured in a scintilla-

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A.  

![Image](http://www.jbc.org/)

B.  

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C.  

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D.  

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Fig. 3. IGF-I-mediated hypertrophy is associated with activation of Erk1/Erk2 MAPK and PI3K pathways. A, cells were pretreated with PD98059 (10 μM) and then incubated with IGF-I for 10 min. Erk1/Erk2 MAPK kinase activity was determined by in vitro kinase assay with MBP as a substrate. B, cells were pretreated with SFM (control) or PD98059 (10 μM) and then treated with IGF-I (50 ng/ml) for the indicated lengths of time. Protein lysates were collected, and phosphorylation of Erk1/Erk2 was determined by immunoblotting with phospho-specific Erk1/Erk2 antibody. Total Erk1/Erk2 was also detected in the same samples using a specific antibody. Relative activation from three separate experiments was quantitated and graphed. C, MCs were pretreated with wortmannin (250 nM) and then treated with IGF-I (50 ng/ml) for the indicated lengths of time. Protein lysates were collected, and phosphorylation of Akt was determined by immunoblotting with a phospho-specific Akt antibody. Also, total Akt was detected in the same samples using a specific antibody. Relative activation from three separate experiments was quantitated and graphed.

Statistical significance was determined by Student’s t test. A result was considered significant if p < 0.05.

RESULTS

In vivo studies demonstrated that IGF-I plays a role in renal hypertrophy (4, 9, 18). However, it is unclear whether IGF-I acts as a proliferative agent for renal cells as it does with many other cells or if IGF-I can directly initiate hypertrophy. Therefore, in this study we examined the effect of IGF-I on cultured mesangial cells (MCs). Hypertrophy was determined as an increase in cell size (without an increase in cell number) and an increase in protein production without an increase in DNA synthesis. Fig. 1A shows that IGF-I treatment (50 ng/ml) of MCs resulted in an increase in cell size as determined by FACS analysis. The effect of IGF-I was comparable to the effect of TGFβ (1 ng/ml), which is known to induce hypertrophy in these cells (19). Treatment of MCs with both IGF-I and TGFβ resulted in an additive hypertrophic effect. FACS analysis of these cells showed that there was no difference in the distribution of cells in the cell cycle after IGF-I or TGFβ treatments compared with SFM control (data not shown).

IGF-I induced hypertrophy in a dose-dependent manner, with 50 ng/ml resulting in a significant increase in both cell size and protein/DNA ratio (Fig. 1B). The maximal effect of IGF-I was equal to or slightly greater than was seen with TGFβ treatment of these cells. Hypertrophy was discernible as early as 8 h after treatment and was detectable for as long as 72 h after exposure of cells to IGF-I (data not shown). Finally, there was a small increase in DNA synthesis following IGF-I treatment but only at concentrations of 100 ng/ml or greater (Fig. 1C).

There are conflicting reports regarding the ability of IGF-I to induce ECM production in renal cells. Previous in vivo experiments have shown that overexpression of IGF-I contributes to hypertrophy but is not sufficient for ECM production and development of fibrosis (7). However, other in vitro studies have shown that IGF-I does contribute to an increase in ECM (20, 21). In our experiments, MCs were treated with IGF-I or TGFβ for 72 h, and the levels of collagen type IV and fibronectin were determined by both Western blotting (Fig. 2A) and immunohistochemistry (Fig. 2B). We found that IGF-I induces an increase in both collagen type IV and fibronectin protein levels although not as robustly as does TGFβ. Interestingly, the amount of IGF-I that was sufficient to stimulate an increase in ECM (at least 100 ng/ml) was double that which was required to induce maximal hypertrophy (50 ng/ml). Similar to hypertrophy, IGF-I and TGFβ together resulted in an additive effect on the levels of ECM proteins.

We next examined the signaling pathways activated by IGF-I in MCs. Using an in vitro kinase assay with myelin basic protein (MBP) as a substrate for Erk1/Erk2 MAPK, we show that IGF-I treatment results in activation of MAPK in MCs (Fig. 3A). This activation was blocked by pretreatment with PD98059, an inhibitor of the Erk1/Erk2 kinase MEK. In addition, we treated MCs with IGF-I for increasing lengths of time and examined phosphorylation of Erk1/Erk2 using a phospho-specific Erk1/Erk2 antibody. Erk1/Erk2 was transiently phosphorylated following addition of IGF-I with maximal activation at 10 min (or less) that was reduced, even to below basal conditions, by 4 h. Pretreatment with PD98059 also blocked phosphorylation of Erk1/Erk2. Total Erk1/Erk2 protein level was determined by direct immunoblotting, and no change in the amount of Erk1/Erk2 protein was found (Fig. 3B).
examined activation of the PI3K pathway following IGF-I treatment in MCs. Fig. 3C shows that IGF-I activated PI3K and resulted in increased production of phosphatidylinositol 3-phosphate. This activation was completely blocked by wortmannin, an inhibitor of PI3K activity. We also treated MCs with IGF-I for increasing lengths of time and examined phosphorylation of Akt, a serine/threonine kinase regulated downstream of PI3K (Fig. 3D). Similar to Erk1/Erk2 phosphorylation, Akt phosphorylation was maximal at 10 min and decreased thereafter. Interestingly, although Akt phosphorylation peaked early, persistent activation was seen at 4 h. In addition, pretreatment with wortmannin blocked only IGF-I-mediated phosphorylation of Akt at 10 min. At 4 h, IGF-I-mediated phosphorylation of Akt was unaffected by wortmannin pretreatment. This suggests a PI3K-independent mechanism of sustained Akt phosphorylation. There were no changes in the levels of Akt protein.

Our experiments thus far indicate that IGF-I-mediated hypertrophy in MCs is associated with activation of Erk1/Erk2 MAPK and PI3K. Therefore, we next determined whether IGF-I-mediated hypertrophy was dependent on activation of either of these pathways. MCs were pretreated with inhibitors of Erk1/Erk2 MAPK (10 μM PD98059), PI3K (250 nM wortmannin), or vehicle alone (MeSO₄), and then IGF-I-mediated hypertrophy was assessed by FACS analysis. Fig. 4A shows that inhibition of Erk1/Erk2 MAPK or PI3K had no effect on IGF-I-induced increase in cell size. Similar results were obtained when protein/DNA ratio was measured. Also, similar results were obtained when PI3K was inhibited with the compound LY294002 (data not shown). We also determined the role of Erk1/Erk2 MAPK and PI3K signaling on IGF-I-mediated increase in ECM proteins collagen type IV and fibronectin. Fig. 4B shows that inhibition of either pathway failed to block the increase in ECM proteins by IGF-I.

Several studies have identified the calcium-regulated serine/threonine phosphatase calcineurin as a potential mediator of hypertrophy (22, 23). Therefore, we examined the role of calcineurin in IGF-I-mediated hypertrophy of MCs. Calcineurin phosphatase activity was determined in response to IGF-I using a highly specific in vitro phosphatase assay (17). The addition of IGF-I to the cells resulted in approximately a 50% increase in calcineurin activity (Fig. 5A). Calcineurin is inhibited by the immunosuppressive compound cyclosporin A. Cyclosporin A enters cells and binds to a group of proteins known as cyclophilins. Cyclosporin A-cyclophilin complexes, in turn, bind to the β subunit of calcineurin, resulting in inhibition of phosphatase activity (24, 25). Due to the specificity of action of cyclosporin A in inhibiting calcineurin, we used this compound to block IGF-I-mediated activation of calcineurin. Calcineurin phosphatase activity was determined in cells pretreated with cyclosporin A (5 μM), PD98059 (10 μM), or wortmannin (250 nM) and then stimulated with IGF-I for 2 h. As expected, pretreatment with cyclosporin A completely blocked calcineurin phosphatase activity, whereas inhibition of either Erk1/Erk2 MAPK or PI3K had no effect (Fig. 5A). We next examined the time course of calcineurin phosphatase activation by IGF-I. Fig. 5B shows that addition of IGF-I resulted in significant activation of calcineurin phosphatase within minutes of treatment that returned to basal levels after 4–6 h. Interestingly, phosphatase activity peaked roughly 2 h after IGF-I treatment, in contrast to the very early maximal phosphorylation of both Erk1/Erk2 MAPK and Akt. Increased calcineurin phosphatase activity has been shown to precede up-regulation of calcineurin protein (26). Accordingly, there was a substantial increase in calcineurin protein within 24 h of IGF-I treatment (Fig. 5C). Calcineurin activity is dependent upon binding both calcium and calmodulin. Therefore, we examined the effect of IGF-I on calmodulin protein levels. There was also a substantial increase in the calcineurin binding protein calmodulin in a time frame similar to the increase in calcineurin protein (Fig. 5C). In contrast, protein levels of Erk1/Erk2 MAPK did not change over the course of the experiment, indicating a selective increase in calcineurin/calmodulin proteins rather than a global increase in total proteins.

To determine if activation of calcineurin is required for IGF-I-induced hypertrophy, MCs were treated with cyclosporin A to
inhibit calcineurin activity prior to addition of IGF-I. Both IGF-I-mediated increase in cell size and increase in protein/DNA ratio were blocked following inhibition of calcineurin.

Moreover, pretreatment of the cells with calcium ionophore A23187 to increase intracellular calcium significantly increased IGF-I-mediated hypertrophy (Fig. 6A). Next we determined if calcineurin was required for IGF-I-mediated induction of ECM. Fig. 6B shows that pretreatment with cyclosporin A blocked IGF-I-mediated increase in both collagen type IV and fibronectin protein levels. Cyclosporin A alone had no effect on either total protein synthesis (reflected by protein/DNA ratio) or on basal levels of collagen type IV or fibronectin.

Inhibition of PI3K with wortmannin only abolished Akt phosphorylation at 10 min and had no effect on IGF-I-mediated Akt phosphorylation at 4 h (Fig. 3D). The lack of inhibition of the delayed Akt activation by wortmannin suggests a PI3K-independent mechanism of sustained Akt activation. Therefore, in Fig. 7A, we examined the role of calcineurin in sustained Akt activation. MC were pretreated with SFM (control), wortmannin (250 nM), or cyclosporin A (5 μM) and then treated with IGF-I for 10 min or 4 h. Phosphorylated Akt was detected by Western blotting with a phospho-specific Akt antibody. At 10 min, wortmannin greatly reduced IGF-I-mediated Akt phosphorylation, whereas cyclosporin A had no effect. After 4 h of IGF-I treatment, Akt phosphorylation was still detectable in control cells, and wortmannin pretreatment does not reduce this phosphorylation. However, pretreatment with...

**Fig. 5.** IGF-I stimulates calcineurin phosphatase activity and increases calcineurin and calmodulin proteins. A, cells were pretreated with SFM, cyclosporin A (5 μM), PD98059 (10 μM), or wortmannin (250 nM) and then stimulated with IGF-I (50 ng/ml) for 2 h. Protein lysates were collected, and calcineurin phosphatase activity was determined as described under “Experimental Procedures.” Data shown are the mean of duplicate assays ± S.E. B, calcineurin phosphatase activity was measured determined following treatment with IGF-I for up to 6 h. *p < 0.01 compared with 0 h treatment. Data shown are the mean of duplicate assays ± S.E. C, cells were treated with IGF-I (50 ng/ml) for up to 24 h and total protein lysates were collected. Calcineurin and calmodulin were detected by direct immunoblotting with specific antibodies. Data shown are representative of at least 3 independent experiments.

**Fig. 6.** Calcineurin is required for IGF-I-mediated hypertrophy and up-regulation of ECM. A, cells were pretreated for 1 h with SFM or cyclosporin A (5 μM) and then incubated with IGF-I for 24 h. Additionally, cells were pretreated with calcium ionophore A23187 (1.5 μM) for 1 h, washed twice with SFM, and then incubated with IGF-I for 24 h. Cells size was determined by mean forward light scatter. Bars represent the mean ± S.E. of four independent experiments. Cells were also analyzed for DNA and protein content and the protein/DNA ratio of triplicate samples ± S.E. was determined. *p < 0.05 from control and **p < 0.05 from IGF-I. B, cells were pretreated for 1 h with SFM or cyclosporin A (5 μM) and then incubated with IGF-I (100 ng/ml) for 72 h. Total cellular proteins were collected and separated by SDS-PAGE. Collagen type IV and fibronectin were detected by direct immunoblotting. Data shown are representative of at least 3 independent experiments.
Fig. 7. IGF-I induces sustained activation of Akt that is mediated by calcineurin. A, cells were pretreated with SFM (control), wortmannin (250 nM), or cyclosporin A (5 μM) and then incubated with IGF-I for 10 min or 4 h. Total protein lysates were collected, and phosphorylated Akt was detected using a phospho-specific antibody. Relative Akt activation was determined by quantitation of phosphorylated Akt normalized by total Akt in each sample. B, cells were pretreated with PD98059 (10 μM) or cyclosporin A (5 μM) and then incubated with IGF-I for up to 4 h. Total protein lysates were collected, and phosphorylated Erk1/Erk2 was detected using a phospho-specific antibody.

Calcineurin Is Required for IGF-I-mediated Hypertrophy

DISCUSSION

In this study, we provide the first evidence that IGF-I plays a direct role in the initiation of hypertrophy of mesangial cells. In addition, we show that IGF-I-mediated hypertrophy is associated with increased expression of the extracellular matrix proteins fibronectin and collagen type IV. Simultaneous exposure of the cells to IGF-I and TGFβ resulted in an additive effect on both hypertrophy and the increase of ECM accumulation, suggesting that these growth factors may act through parallel pathways. IGF-I is a well-characterized mitogen, and it has been speculated that the role of IGF-I in renal hypertrophy is due to its effect on cell proliferation. However, in our experiments, no change in the cell cycle was seen and no significant change in DNA synthesis was observed at the same dose of IGF-I required to induce maximal hypertrophy. At higher doses of IGF-I, there was an increase in DNA synthesis, consistent with other reports in the literature (29, 30).

IGF-I has been shown to signal via Erk1/Erk2 MAPK and PI3K in many different cell types (11). However, the precise...
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role of each signaling pathway in mediating the biological effects of IGF-I remains to be characterized. Although Erk1/Erk2 MAPK was activated by IGF-I in our studies, it does not appear to be required for induction of hypertrophy. It is still possible that the MAPK pathway might be important for other cellular responses to IGF-I, including migration, modulation of DNA synthesis, and cell survival. Treatment of MCs with IGF-I also resulted in the activation of PI3K and Akt kinase. Interestingly, the serine/threonine kinase Akt is activated by IGF-I in a bi-phasic manner. IGF-I induced rapid activation of Akt (10 min) that was inhibited by wortmannin, demonstrating that this activation is mediated by PI3K. However, wortmannin had no effect on the delayed phase of Akt activation, indicating a PI3K-independent mechanism of activation. Inhibition of PI3K by wortmannin or LY294002 had no effect on IGF-I-induced hypertrophy, demonstrating that PI3K is not required for this biological effect of IGF-I. However, the sustained activation of Akt was inhibited by cyclosporin A, indicating that activation of calcineurin by IGF-I is responsible for the sustained Akt activation in MCs. Our data support other reports in the literature that calcineurin activation can act in concert with other signaling pathways (31, 32). The consequences of sustained Akt activation by calcineurin and the role of Akt in IGF-I-mediated hypertrophy remain to be evaluated.

Our data show that IGF-I treatment of MCs not only increases the levels of calcineurin protein, but also calmodulin. As calcineurin activation is dependent upon availability of calmodulin (33), one possible consequence of increased calmodulin is sustained activation of other calcium/calmodulin pathways and/or priming of the calcineurin/calmodulin system for activation by other signals. Interestingly, the increase in both calcineurin and calmodulin occurs rapidly following IGF-I treatment, particularly in comparison to the global increase in protein reflected in the protein/DNA ratio. This suggests that the IGF-I-mediated increase in calcineurin and calmodulin protein expression is a specific action of IGF-I and not merely a consequence of the generalized increase in protein synthesis associated with hypertrophy. However, a more dynamic assay of protein synthesis such as incorporation of [35S]methionine into proteins would more accurately address the contribution of global protein synthesis to the increased expression of calcineurin and calmodulin. Moreover, our data do not address the contribution of protein degradation to the modulation of calcineurin/calmodulin levels.

Interestingly, calcineurin activation occurred fairly late after IGF-I treatment. Both Erk1/Erk2 MAPK and Akt are phosphorylated within minutes of IGF-I addition and peak activation has subsided in less than 1 h. Calcineurin activity appears to peak ~2 h following addition of IGF-I and returns to baseline by 4–6 h. Additionally, calcineurin activation precedes a significant increase in calcineurin and calmodulin protein levels, as well as induction of hypertrophy by IGF-I. Our data show that this increase in phosphatase activity is in fact required for IGF-I-mediated hypertrophy. Inhibition of calcineurin with cyclosporin A completely blocks IGF-I-mediated activation of calcineurin phosphatase activity and IGF-I-mediated hypertrophy and ECM accumulation. These effects of cyclosporin A on matrix protein accumulation were specific, because the compound had no effect on total protein synthesis or on the levels of other proteins such as Erk1/Erk2. Of interest is the finding in Fig. 6A that increased intracellular calcium due to treatment with ionophore A23187 did not induce hypertrophy. This is consistent with the observation, reported by Musaro et al. (34), that calcium alone is actually toxic to some cells. Instead, there appears to be a requirement for an additional IGF-I-mediated signal to generate a hypertrophic response. One possibility is cross-talk with other signaling pathways such as Akt (35, 36).

Several proteins have been identified as targets of calcineurin-mediated dephosphorylation, including NFAT, MEF2A and -2D, and GATA (15, 33). Our data demonstrate that calcineurin is important in IGF-I-mediated hypertrophy. Therefore, calcineurin substrates may also be critical signaling molecules that mediate hypertrophy in MCs. IGF-I induces nuclear localization of NFATc1, which is dependent upon calcineurin activity. Therefore, NFATc1 may be important in transcriptional regulation of hypertrophic response genes, including ECM genes and possibly even of calcineurin and calmodulin. It remains to be seen whether other calcineurin substrates also play a role in the regulation of genes responsible for cell hypertrophy.

The demonstration that IGF-I activates a calcium-dependent serine/threonine phosphatase in mesangial cells introduces a new paradigm in IGF signaling. In addition to its role as a mitogen, requiring primarily the MAPK and PI3K signaling pathways, our work and the work of other groups in this area show that IGF-I elicits relevant biological responses via calcium-dependent signaling pathways, including, but possibly not limited to, calcineurin phosphatase. Similarly, this study demonstrates another potential target tissue for the action of calcineurin. Discovering how growth factors such as IGF-I activate calcineurin and what factors downstream of calcineurin are required for inducing hypertrophy will be important areas for further research. Furthermore, calcineurin represents an intriguing new pathway with substantial relevance in renal disease. Treatment of animal models of cardiac hypertrophy with inhibitors of calcineurin have been successful at preventing hypertrophy (22, 26, 32); therefore, it is possible that calcineurin inhibitors may also be of therapeutic value in renal diseases characterized by hypertrophy.

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