Role of Mammalian Target of Rapamycin Signaling in Compensatory Renal Hypertrophy

Jian-Kang Chen,* Jianchun Chen,* Eric G. Neilson,*† and Raymond C. Harris*
Departments of *Medicine and †Cell Biology, Vanderbilt University School of Medicine, and the Department of Veterans Affairs, Nashville, Tennessee

Loss of functioning nephrons stimulates the growth of residual kidney tissue to augment work capacity and maintain normal renal function. This growth largely occurs by hypertrophy rather than from hyperplasia of the remaining nephrons. The signaling mechanisms that increase RNA and protein synthesis during compensatory renal hypertrophy are unknown. This study found that the remaining kidney hypertrophied 42% by 16 d after unilateral nephrectomy (UNX) in DBA/2 mice. Immunoblotting analysis revealed increased phosphorylation of the 40S ribosomal protein S6 (rpS6) and the eukaryotic translation initiation factor (eIF) 4E-binding protein 1 (4E-BP1), the two downstream effectors of the mammalian target of rapamycin (mTOR). The highly specific mTOR inhibitor rapamycin blocked UNX-increased phosphorylation of both rpS6 and 4E-BP1. UNX increased the content of not only 40S and 60S ribosomal subunits but also 80S monosomes and polysomes in the remaining kidney. Administration of rapamycin decreased UNX-induced polysome formation and shifted the polysome profile in the direction of monosomes and ribosomal subunits. Pretreatment of the mice with rapamycin inhibited UNX-induced hypertrophy. These studies demonstrate that activation of the mTOR signaling pathway in the remaining kidney after UNX plays an essential role in modulating RNA and protein synthesis during development of compensatory renal hypertrophy.

I n response to reduction in the number of functioning nephrons, the remaining nephrons increase their RNA and protein synthesis with minimal alterations in DNA replication (1). These changes in protein synthesis increase residual kidney size but not numbers of existing renal cells, presumably to augment functional capacity as compensation for the nephron loss (2,3). In contrast to hyperplasia, this type of growth is called compensatory renal hypertrophy. In some cases, renal hypertrophy may actually be an excessive or maladaptive response that fosters further nephron damage, progressive decline of renal function, and ultimately ESRD. Judicious attenuation of hypertrophy with drugs may limit the progression of renal injury in various forms of chronic kidney disease (2–6). Renal hypertrophy is modulated by a cell cycle–dependent mechanism whereby cyclin-dependent kinase (CDK) 4/cyclin D is activated without a subsequent engagement of CDK2/cyclin E, which is typically regulated by TGF-β (7–9) and the CDK inhibitor p27kip1 (10,11). The cell cycle thus is arrested in late G1 without progression into S phase, resulting in hypertrophy instead of hyperplasia (8,9,12). To date, however, the mechanisms that mediate the increased RNA and protein synthesis seen in compensatory renal hypertrophy are not understood.

Recent studies have demonstrated that the ubiquitously expressed mammalian target of rapamycin (mTOR) is an important regulator of RNA and protein synthesis in mammalian cells (13,14). mTOR functions through two major downstream effectors in the regulation of protein synthesis and control of cell size. One effector is the eukaryotic translation initiation factor (eIF) 4E-binding protein 1 (4E-BP1). mTOR-dependent phosphorylation of 4E-BP1 releases eIF4E, initiating cap-dependent de novo translation of various species of mRNA (15,16). Another set of downstream effectors of mTOR are the serine/threonine protein kinases, S6 kinase 1 (S6K1) and S6 kinase 2 (S6K2), both of which are responsible for phosphorylation of the 40S ribosomal protein S6 (rpS6) (17). mTOR activation of S6K results in phosphorylation of rpS6, leading to recruitment of the 40S ribosome subunit and translation of 5′-terminal oligopyrimidine tract (5′TOP) mRNA (18). These 5′TOP mRNA encode primarily ribosomal proteins and other components of the translational apparatus (19). Thus, by controlling S6K activity, mTOR also regulates the abundance of translational machinery. Under favorable growth conditions, mTOR is activated and S6K and 4E-BP1 are phosphorylated, leading to new RNA and protein synthesis. Because renal hypertrophy after nephron loss is characterized by increased RNA and protein synthesis with minimal DNA replication and cell division (1), we used a mouse model of unilateral nephrectomy (UNX) to examine whether the mTOR signaling pathway is causally involved in the initiation of compensatory renal hypertrophy.
Materials and Methods

Chemicals and Antibodies
Antibodies against phospho-rpS6, total 4E-BP1, or phospho-4E-BP1 (Thr37, Thr46, Ser65, or Thr70) were from Cell Signaling Technology (Beverly, MA). Antibodies to β-actin and other chemical reagents were from Sigma (St. Louis, MO). Rapamycin was provided by Wyeth-Ayerst Research (Princeton, NJ).

Animal Care and Surgical Procedures
Animals were housed at the Vanderbilt MRBII veterinary facility (MRBII Rm 882). Animal care and experimental procedures complied with the regulations of Vanderbilt University’s Institutional Animal Care and Usage Committee. Compensatory renal hypertrophy was induced by right UNX primarily in male DBA/2 mice (8 to 10 wk of age). UNX was performed through a flank incision, sparing the adrenal gland, under anesthesia using sodium pentobarbital (50 mg/kg intraperitoneally). Left kidneys of right sham-nephrectomized (SHAM) mice were used as controls. SHAM consisted of anesthesia, flank incision, delivery of the right kidney through the incision, and return to the retroperitoneum.

Immunoblotting Analysis
Immunoblotting procedures were performed as described previously (20). Briefly, left kidneys were decapsulated, cut into eight pieces, and washed twice with ice-cold PBS, followed by homogenization in a lysis buffer that contained 0.5% Nonidet P-40, 50 mM NaCl, 10 mM Tris-HCl (pH 7.4), 2 mM EDTA, 2 mM EGTA, 0.5% sodium deoxycholate, 0.1% SDS, 100 µM Na3VO4, 10 mM NaF, 30 mM sodium pyrophosphate, 1 mM PMSE, 10 µg/ml aprotinin, and 10 µg/ml leupeptin (20). Whole-kidney lysates were clarified at 10,000 × g for 15 min at 4°C, and protein concentrations were determined by the Bradford protein assay (Bio-Rad Laboratories, Hercules, CA). Equal amounts of protein were loaded onto 7 to 15% SDS-PAGE, transferred onto polyvinylidene difluoride membranes, probed with the indicated primary antibody and the appropriate secondary antibody conjugated with biotin, and incubated with preformed avidin-biotin-horseradish peroxidase complex using a commercially available kit (ABC kit; Pierce, Rockford, IL), and the immune complexes were detected by a peroxidase-catalyzed enhanced chemiluminescence detection system (ECL; Amersham Biosciences, Piscataway, NJ).

Polysome Profile Analysis
Adult DBA/2 mice were administered either rapamycin or vehicle alone. After 2 h, the animals were subjected to right UNX or SHAM surgery. Left kidneys were homogenized in a 7-ml Dounce homogenizer, followed by a 10-fold dilution. DNA determination was performed in triplicate as described previously (21). Briefly, aliquots of each homogenate were incubated in a 96-well plate at 37°C for 1 h. After addition of 100 µl of 1.0 µg/ml bisbenzimidezole fluorescent dye Hoechst 33258 (Sigma), the samples were read at excitation λ 360 nm, emission λ 460 nm using a CytoFluo II spectrophotofluorimeter (PerSeptive Biosystems, Cambridge, MA). Aliquots of the same homogenates were used to determine protein concentration by the Bradford protein assay (Bio-Rad Laboratories). The protein/DNA ratios were calculated, and data were presented as percentage increases compared with sham-operated mice.

Statistical Analyses
Data are presented as means ± SEM for at least three separate experiments (each in triplicate or duplicate). An unpaired t test was used for statistical analysis, and ANOVA and Bonferroni t test were used for multiple group comparisons. P < 0.05 was considered statistically significant.

Results
Identification of DBA/2 Mice as the Most Suitable Strain for Investigating Renal Hypertrophy
The susceptibility of mouse kidney to sclerosis in response to nephron loss is strain dependent (22); therefore, we initially compared the development of renal hypertrophy in response to UNX in the following mouse strains: C57BL/6J, 129/SvJ, B6/129S6F1, and DBA/2. It is well established that UNX-induced increases in residual kidney weight are largely due to increases in the protein content of cells along the nephron, primarily in the proximal tubule, rather than either increased cell proliferation or increased water content, such as is seen with pregnancy (1–3,23,24); therefore, the percentage increase in kidney/body weight ratio in comparison with sham-operated mice has been used extensively to indicate the degree of renal hypertrophy, particularly in the case of UNX. We found that DBA/2 mice showed the least individual variability and exhibited a relatively high degree of hypertrophy. With adult DBA/2 mice, 6 h, 24 h, 4 d, and 16 d after UNX, the remaining kidney hypertrophied 8, 15, 28, and 42%, respectively (Figure 1). Accordingly, we used DBA/2 mice (male, 8 to 10 wk of age) for our subsequent studies.

UNX Stimulates Phosphorylation of rpS6 in the Remaining Kidney
Previous studies in rats reported conflicting results regarding S6 kinase activation in the remaining kidney after UNX (25,26). In our study, we found that compared with SHAM mice, UNX significantly increased the levels of rpS6 phosphorylation in the remaining kidney within 30 min; rpS6 phosphorylation peaked at 24 h and remained elevated for up to 4 d, the longest time examined (Figure 2, top). Figure 2, bottom, reflects stripping and reprobing of the same blot by a β-actin antibody.

Rapamycin Inhibits UNX-Induced Phosphorylation of rpS6
Rapamycin inhibits mTOR kinase activity by binding to the FRB domain of mTOR in the form of a rapamycin-FKBP12 complex (FKBP12 is a 12-kD protein that serves as the intracellular receptor for rapamycin) (27). The discovery of rapamycin as a highly specific mTOR inhibitor has made it possible to explore and delineate the role and signaling mechanism of mTOR (13–18,27). Because S6K1 and S6K2 are downstream targets of mTOR that are responsible for rpS6 phosphorylation in the cell (17,28,29), we used rapamycin to examine whether mTOR mediates UNX-induced rpS6 phosphorylation. Our results show that UNX increased S6 kinase activity, indicated by elevated phosphorylation levels of rpS6, that was completely blocked by...
administration of rapamycin, suggesting that mTOR is upstream of S6 kinase activation during the hypertrophic response to UNX (Figure 3).

**UNX-Stimulated Phosphorylation of 4E-BP1 at Multiple Sites Is Inhibited by Rapamycin**

In addition to S6K, another downstream target of mTOR is 4E-BP1, which is a translational inhibitor in the cell (16). Activation of mTOR phosphorylates 4E-BP1 at multiple sites in a characteristically hierarchical manner (16,30). As schematically indicated by the arrows in Figure 4A, phosphorylation at Thr46 is required for phosphorylation at Thr37, which is required for phosphorylation at Thr70, and phosphorylation at Thr70 and Ser101 is required for phosphorylation at Ser65. Phosphorylation of Ser112, at the extreme C-terminus, is also required for dissociation of 4E-BP1, whereas Ser83 seems to be phosphorylated at baseline. Hyperphosphorylated 4E-BP1 dissociates from and consequently activates eIF4E, the translation initiation factor that binds the 5' cap structure of mRNA and regulates cap-dependent translation (16,30).

4E-BP1 migrates anomalously when subjected to SDS-PAGE (apparent relative molecular mass 20 to 24 kD) as a result of its high content of proline and glycine (31). When kidney homogenates were probed by a polyclonal antibody that recognizes total 4E-BP1 regardless of its phosphorylation state, 4E-BP1 migrated at least three to four bands, denoted as shown in Figure 4B, representing 4E-BP1 protein phosphorylated to varying extents, similar to previous reports (15,32). Because the shift to species of higher apparent relative molecular mass is indicative of increased phosphorylation (15,32), these data indicate that UNX resulted in substantial increases in 4E-BP1 phosphorylation within 6 h (the earliest time point examined), and the phosphorylation remained elevated through 24 h (the longest time tested; Figure 4B). The increased 4E-BP1 phosphorylation was inhibited by pretreatment with rapamycin 2 h before surgery, with the 24-h group that was treated with rapamycin being less inhibited (Figure 4B), presumably as a result of the diminished effect of the drug 26 h after administration.

Figure 1. Time course of compensatory renal hypertrophy in DBA/2 mice. Male DBA/2 mice (10 wk old) were subjected to unilateral nephrectomy (UNX) or SHAM surgery as described in the Materials and Methods section. UNX-induced compensatory renal hypertrophy was assessed by increases in kidney/body weight ratio compared with SHAM mice. Values are means ± SEM (n = 4 for each time point group of UNX or SHAM). *P < 0.01; **P < 0.001 versus their corresponding SHAM groups at each time point. No significant difference was seen among the SHAM groups at different time points (P > 0.05).

Figure 2. UNX-induced phosphorylation of the 40S ribosomal protein S6 in the remaining kidney. Adult DBA/2 mice were subjected to right UNX (U) or SHAM surgery (S) as described in the Materials and Methods section. At the times indicated, the animals were killed and their left kidney lysates were subjected to immunoblotting analysis with an antibody that recognizes only phosphorylated ribosomal protein S6 (phospho-rpS6), and the blot was stripped and reprobed with an antibody to β-actin to ensure equal loading. Each sample was from pooled kidney lysates from three mice for each group. Shown is a representative blot of three separate experiments with similar results.

Figure 3. Rapamycin inhibition of UNX-induced phosphorylation of rpS6. Adult DBA/2 mice were administered either rapamycin (Rapa; 4 mg/kg body wt intraperitoneally) or vehicle (Veh) alone 2 h before right UNX or SHAM surgery and were killed 1 or 4 d later. For the 4-d time period groups, the mice were administered rapamycin or vehicle once daily. (Bottom) Equal loading by immunoblotting with a β-actin antibody. Each sample was from pooled kidney lysates from three mice for each group. Shown are representative blots from one of three separate experiments with similar results.
pho-specific antibodies, as indicated in Figure 4, C through F. The phospho-Thr46 antibody recognizes all four 4E-BP1 species (Figure 4C), because in the four phosphorylation states from α through δ, Thr46 is always phosphorylated, owing to the ordered phosphorylation of 4E-BP1 is Thr46 → Thr37 → Thr70 → Ser65, as depicted in Figure 4A. The phospho-Thr37 antibody recognizes mainly the β, γ, and δ bands (Figure 4D), and the phospho-Thr70 antibody recognizes γ and δ species (Figure 4E), whereas the phospho-Ser65 antibody recognizes only the δ band (Figure 4F), the superphosphorylated species (15,31,32).

In kidneys that underwent hypertrophy, phosphorylation of 4E-BP1 was detected at multiple sites, including Thr37, Thr46, Thr70, and Ser65. Moreover, phosphorylation of Ser65 and Thr70 was completely prevented by rapamycin, with phosphorylation of Thr46 being less inhibited and that of Thr37 the least inhibited (Figure 4, C through F). These observations are consistent with previous descriptions (33), suggesting that phosphorylation of Thr37 and Thr46 is relatively resistant to rapamycin, whereas phosphorylation of Ser65 and Thr70 is rapamycin sensitive.

**Polysome Profile Analysis after UNX**

The characteristic changes of compensatory renal hypertrophy are increases in both protein and RNA synthesis, which are not only the components of the translational machinery but also the functional products of this machinery. Protein synthesis occurs in a multimeric structure, the polysome, which consists of multiple ribosomes arrayed along the length of the mRNA (34). Polysomal profiles contain information not only about polysomes but also about other translation machinery components, including the small ribosomal subunits (40S), large ribosomal subunits (60S), and monosomes (80S). Changes in this profile are indicative of changes in translation activity (34).

Accordingly, we examined the polysome profile in the remaining kidney of SHAM or UNX mice that were pretreated with rapamycin or vehicle alone. We found that UNX increased not only the content of 40S and 60S ribosomal subunits but also that of 80S monosomes and polysomes in the remaining kidney (Figure 5, shaded areas), indicating that UNX initiated increases in biosynthesis of RNA and protein as well as biogenesis of protein synthesis machinery. These increases seemed to be mTOR dependent, because rapamycin reduced UNX-stimulated polysome formation and shifted the polysome profile toward the direction of monosome and ribosomal subunits (Figure 5).

Rapamycin Inhibits Development of Compensatory Renal Hypertrophy Induced by UNX

For examining the potential role of mTOR in renal hypertrophy, mice were given rapamycin daily (4 mg/kg body wt intraperitoneally, a clinically relevant dose of rapamycin [35,36]), with the first injection being 2 h before the surgery. As shown in Figure 6A, a single dose of rapamycin almost completely prevented development of renal hypertrophy 24 h after UNX compared with the vehicle-treated group. Daily injection of rapamycin for 4 successive days after UNX also significantly inhibited the hypertrophy.
Discussion

The mechanisms that regulate initiation of renal hypertrophy in response to reduction of functioning nephrons are fundamental to understanding the biology of the kidney. This study demonstrates that the mTOR signaling is activated in the remaining kidney in response to UNX and indicates a central role for activation of mTOR signaling in the development of compensatory renal hypertrophy. Inhibition of mTOR activity not only reduced predicted increases in the polysome content and shifted the polysome profile toward the direction of the monosome but also markedly prevented renal hypertrophy after UNX. Our studies therefore demonstrate that activation of mTOR signaling by UNX is an essential early regulatory mechanism that initiates increased RNA and protein synthesis in hypertrophic renal growth. This represents the first identification of an early signaling pathway that mediates initiation of compensatory renal hypertrophy.

Because activation of mTOR is easily detected 30 min after UNX, the upstream signal mediators of mTOR need to be engaged even earlier. In this respect, growth factor activation of PDK1/Akt has been implicated to be upstream of mTOR, although this has not yet been established in the hypertrophying kidney. In addition, tuberous sclerosis complex (TSC), characterized by multiple hamartomas and caused by mutation in either one of the two tumor suppressor genes TSC1 and TSC2, is characterized by constitutive activation of mTOR (37,38).

Recent studies indicate that TSC2 is the direct phosphorylation target of Akt, and this phosphorylation eliminates the inhibitory effect of TSC1-TSC2 complex on mTOR activity (39). More recently, the small GTP-binding protein Rheb (Ras homolog enriched in brain) was identified to be downstream of the TSC1-TSC2 heterodimer but upstream of mTOR (40). Besides growth factors, other factors, including cellular energy and nutrient availability, may also play a role in regulation of mTOR activity (41,42).

Our analysis of the polysome profile during hypertrophy revealed that rapamycin pretreatment increased the large (60S) subunit peak but decreased the small (40S) subunit peak compared with vehicle-pretreated UNX controls. One explanation for this observation is that rapamycin inhibited selectively the biogenesis of the 40S subunit, although the biogenesis of the 60S subunit might also have been inhibited to a lesser extent, resulting in an accumulation of the 60S subunits and consequently inhibiting polysome formation and protein synthesis. This notion suggests that mTOR-independent mechanisms may be involved in the production of the 60S subunits. Alternatively, inhibition of protein synthesis might have occurred by inhibiting the last step of polysome formation, recruitment of the 60S subunit, leading to the accumulation of 60S subunits. These alterations induced by rapamycin are consistent with the role of mTOR, through S6K and 4E-BP1, in the recruitment of ribosomal subunits in the process of polysome formation and accelerated protein synthesis, although the precise details remain unclear (15–19). Further investigation of this intriguing observation may provide new insights into the mechanisms of renal hypertrophy and the regulatory role of mTOR in protein synthesis.

Increased mTOR activity not only stimulates ribosome biogenesis and protein synthesis but also modulates a range of cellular activities that are essential for cell growth, including actin cytoskeleton organization, inhibition of autophagic protein degradation, nutrient transporter turnover, and protein kinase C signaling (13,16). Furthermore, mTOR may play an important role in regulation of other growth regulatory elements such as cyclin D, Rb, p21<sup>cip1</sup>, and p27<sup>kip1</sup> (43,44). It is
this regulation of such a broad range of growth-related processes that places mTOR in a central position of controlling cell growth and size (13,16). However, the underlying mechanisms of renal hypertrophy may not be the same in all situations. For instance, pregnancy-associated increases in kidney size are reversible and most likely due primarily to an increase in water content rather than cellular hypertrophy (45). In contrast, the increase in protein content during kidney hypertrophy after UNX or high-protein diets is due to increased protein synthesis. Both reduced proteolysis and accelerated protein synthesis contribute to the increased protein content in kidneys that hypertrophy with poorly controlled diabetes as well as in NH4Cl-induced chronic acidosis (23,24). It will be interesting to examine the potential role for mTOR in regulation of renal hypertrophy under these conditions, because mTOR-dependent signaling not only promotes protein synthesis but also inhibits autophagic protein degradation (46).

In cultured cell systems, TGF-β converts angiotensin II- or EGF-induced hyperplasia to hypertrophy by inhibiting mitogen-induced hyperphosphorylation of pRB (the product of the retinoblastoma gene) (7,8). Of interest, both diabetes- and UNX-induced renal hypertrophy is regulated by a cell cycle–dependent mechanism in which CDK4/cyclin D is activated without a subsequent full activation of CDK2/cyclin E, thus arresting the cell cycle in late G1 (12,47); in contrast, NH4Cl-induced hypertrophy seems to represent an imbalance between rates of protein synthesis and degradation and has been characterized as cycle independent (2,12,47). In addition to regulation by phosphorylation, the activity of cyclin/Cdk complexes is attenuated by the binding of CDK inhibitors such as p21Cip1 and p27Kip1 (48). There is accumulating evidence that renal cell hypertrophy in response to angiotensin II is dependent on p27Kip1-mediated cell-cycle arrest at the G1 phase such that an enlarged cell does not divide (10,11,49,50). These studies of cell cycle–dependent mechanisms are complementary to our finding of a role for mTOR-mediated signaling in compensatory renal hypertrophy, because the studies of CDK/cyclins and CDK inhibitors explain why cells undergo hypertrophy rather than hyperplasia. Our studies delineate pathways that mediate the increased RNA and protein synthesis during hypertrophic growth.

In summary, this study provides the first definitive evidence that mTOR is activated in the remaining kidney after UNX. In addition, we determined that renal ablation increased the content of not only polysomes and monosomes but also 40S and 60S ribosomal subunits in the remaining kidney, indicating initiation of mRNA translation and enhanced capacity of protein synthesis. Furthermore, rapamycin pretreatment reduced UNX-induced polysome formation and shifted the polysome profile toward the monosome direction. These data confirm that initiation of ribosome biogenesis and increased RNA and protein synthesis induced by renal ablation is mediated by an mTOR-dependent signaling pathway. Moreover, our data demonstrate that when the mTOR signaling pathway is blocked, UNX-induced renal hypertrophy is inhibited. Taken together, these studies indicate that activation of mTOR signaling is essential in the initiation of ribosome biogenesis and in-

Figure 6. Inhibition of rapamycin on UNX-induced compensatory renal hypertrophy. Ten-week-old DBA/2 mice were pre-treated with rapamycin (Rapa) or vehicle alone for 2 h before right UNX or SHAM surgery. (A) UNX-induced compensatory renal hypertrophy was assessed by increases in kidney/body weight ratio compared with SHAM mice. Values are means ± SEM (n = 6 for each group). *P < 0.01; **P < 0.001. Additional studies were performed to compare the mTOR activities indicated by rpS6 phosphorylation levels (B) as well as protein/DNA ratios (C) in renal cortex versus medulla. **P < 0.001; *P < 0.05.
creased RNA and protein synthesis in compensatory renal hypertrophy.

Acknowledgments

This work was supported by funds from National Institutes of Health Grants DK38226 (J.-K.C. and R.C.H.), DK51265 (R.C.H.), and DK46282 (E.G.N.) and the Department of Veterans Affairs.

We thank Wyeth-Ayerst Research (Princeton, NJ) for generously providing rapamycin.

References


14. Fingar DC, Salama S, Tsou C, Harlow E, Blenis J: Mammalian cell size is controlled by mTOR and its downstream targets S6K1 and 4EBP1/eIF4E. *Genes Dev* 16: 1472–1487, 2002


34. Wettstein FO, Staehelin T, Noll H: Ribosomal aggregate...