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Mammalian Target of Rapamycin Is a Critical Regulator of Cardiac Hypertrophy in Spontaneously Hypertensive Rats

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Abstract—Evidence exists that protein kinase C and the mammalian target of rapamycin are important regulators of cardiac hypertrophy. We examined the contribution of these signaling kinases to cardiac growth in spontaneously hypertensive rats (SHRs). Systolic blood pressure was increased ($P<0.001$) at 10 weeks in SHRs versus Wistar-Kyoto controls (162±3 versus 128±1 mm Hg) and was further elevated ($P<0.001$) at 17 weeks in SHRs (184±7 mm Hg). Heart:body weight ratio was not different between groups at 10 weeks but was 22% greater ($P<0.01$) in SHRs versus Wistar-Kyoto controls at 17 weeks. At 10 weeks, activation of Akt and S6 ribosomal protein was greater ($P<0.01$) in SHRs but returned to normal by 17 weeks. In contrast, SHRs had protein kinase C activation only at 17 weeks. To determine whether mammalian target of rapamycin regulates the initial development of hypertrophy, rats were treated with rapamycin (2 mg/kg per day IP) or saline vehicle from 13 to 16 weeks of age. Rapamycin inhibited cardiac mammalian target of rapamycin in SHRs, as evidenced by reductions ($P<0.001$) in phosphorylation of S6 ribosomal protein and eukaryotic translation initiation factor-4E binding protein 1. Rapamycin treatment also reduced ($P<0.001$) heart weight and hypertrophy by 47% and 53%, respectively, in SHRs in spite of increased ($P<0.001$) systolic blood pressure versus untreated SHRs (213±8 versus 189±6 mm Hg). Atrial natriuretic peptide, brain natriuretic peptide, and cardiac function were unchanged between SHRs treated with rapamycin or vehicle. These data show that mammalian target of rapamycin is required for the development of cardiac hypertrophy evoked by rising blood pressure in SHRs. **(*Hypertension*. 2009; 54:1321-1327.)**

Key Words: heart ■ blood pressure ■ signal transduction ■ hypertrophy ■ mTor

Over the last decade, much research has focused on identifying the signaling pathways that regulate cardiac hypertrophy. Among these pathways, protein kinase C (PKC) and the mammalian target of rapamycin (mTOR) have emerged as potentially important regulators of cardiac hypertrophy.[1]

PKC is a family of serine-threonine kinases consisting of 11 isoforms in the heart.[2] Studies using transgenic mice with cardiac specific overexpression of PKC βII or ε, and mice with overexpression of peptide activators of PKC δ and ε, have reported that these isoforms regulate pathological (PKC βII) and/or physiological cardiac hypertrophy (PKC δ and ε).[3–6] Similarly, humans with hypertrophy and heart failure exhibit activation of PKC-α and -βII.[7]

Signaling through components of the mTOR pathway is an important regulator of normal cardiac growth and pathological hypertrophy. For example, overexpression of phosphoinositide 3-kinase in mice results in Akt activation and increased heart size, whereas overexpression of dominant-negative phosphoinositide 3-kinase leads to decreased Akt activation and reduced heart size.[8] Other studies using cardiac-specific overexpression of Akt report that development of both physiological and pathological hypertrophy is correlated with the degree of Akt activation.[9,10] Human studies examining components of mTOR signaling during hypertrophy or heart failure are scarce, and what data exists is conflicting. For example, it has been reported that implantation of a left ventricular assist device in patients with heart failure resulted in cardiac improvements (reduced left ventricular end-diastolic dimensions and apoptosis), associated with a reduction in phosphorylated (p)-Akt.[11] In contrast, a more recent study stated that hypertensive patients without heart failure had higher p-Akt than patients with heart failure.[12] Therefore, the role of the mTOR signaling pathway during human hypertrophy and heart failure remains unclear.

In the present study, we tested the hypothesis that PKC and mTOR contribute to cardiac hypertrophy that develops in spontaneously hypertensive rats (SHRs). We chose the SHR because these animals model human hypertension and cardiac growth. In this regard, these rats are normotensive at 6 weeks of age but develop hypertension and cardiac hypertrophy at ≈12 weeks of age and heart failure by ≈24 months.[13,14] Data provided herein show that signaling via mTOR, but not PKC, is increased in SHRs during the development of cardiac hypertrophy (ie, at 10 weeks). Furthermore, when mTOR was

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From the College of Health (W.S., H.-y.L., E.H., S.L., J.D.S., T.J.) and Divisions of Cardiology (S.E.L.), and Human Molecular Biology and Genetics (S.S., E.D.A.), University of Utah, Salt Lake City, Utah.

Correspondence to Thunder Jalili, 250 S 1850 E, Room 214, University of Utah, Salt Lake City, UT 84112. E-mail Thunder.Jalili@utah.edu

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inhibited using rapamycin, cardiac hypertrophy was attenuated independent of changes in blood pressure. These data show clearly that mTOR is required for the initiation and full development of cardiac hypertrophy evoked by rising blood pressure in SHRs.

Methods

Please see the online Data Supplement at http://hyper.ahajournals.org for a detailed description of the methods and experimental groups.

Animals

All of the protocols were approved by the University of Utah Institutional Animal Care and Use Committee. Six-week–old male SHRs (n=42) and Wistar Kyoto rats (WKYs; n=24) were purchased from Harlan (Indianapolis, IN) and housed in the University of Utah Comparative Medicine Center under standard conditions (12:12-hour light:dark cycle) and were allowed free access to food and water. Rapamycin was purchased from LC Laboratories.

Blood Pressure

Blood pressure was measured using a fluid-filled catheter placed into the caudal artery of rats anesthetized with 2% to 5% isoflurane.[15,16] After rats regained consciousness, blood pressure was measured over 20 cardiac cycles.

RNA Extraction and Quantitative RT-PCR

Total RNA was extracted from the left ventricle (LV) using TRIzol reagent (Invitrogen) and purified using the RNeasy total RNA isolation kit (Qiagen). RT-PCR was done as detailed by Boudina et al.[17]

Tissue Homogenization and Western Blotting

Homogenization of the LV, electrophoresis, and transfer of proteins to polyvinylidene difluoride membranes were done as we have described previously.[18,19] Western blots were verified in duplicate if no significant differences were observed or triplicate if significant differences were present.

Myocardial Function

Cardiac function was determined in a subset of SHRs after 3 weeks of rapamycin (2 mg/kg IP; n=5) or vehicle (saline; n=5) treatment using echocardiography.[15]

Statistical Analysis

An ANOVA was used to detect differences among groups using SPSS version 11 for Macintosh (SPSS Inc). When a significant P value was obtained ($P<0.05$), post hoc procedures were performed using the least significant difference analysis to identify individual group differences. Results are presented as mean±SE.

Results

PKC, mTOR, Cardiac Mass, and Blood Pressure in 10-Week– and 17-Week–Old SHRs

At 10 weeks of age, heart:body weight ratio was similar in WKYs versus SHRs (Table 1). In contrast, cardiac hypertrophy was present in 17-week–old SHRs, as evidenced by increased heart:body weight ratio versus WKYs (Table 1). Systolic blood pressure was 26% higher in 10-week–old SHRs compared with WKYs and 33% greater in 17-week–old SHRs versus WKYs (Table 1).

There were no differences detected in total protein expression of PKC-α, PKC-βII, PKC-δ, or PKC-ε in SHRs versus WKYs at either age (Figure 1A; data not shown for PKCδ). Levels of p-PKC-ε were also similar at both ages of SHRs and WKYs (Figure 1A). p-PKC-δ was not detected in the myocardium of SHRs or WKYs. This might be because of a lack of specificity of the primary antibody against rat heart p-PKC-δ rather than an absence of p-PKC-δ. There was no change in p-PKC-α/βII in 10-week–old SHRs compared with their age-matched controls. However, 17-week–old SHRs had an ≈80% increase in p-PKC-α/βII versus WKYs (Figure 1A). To control for any possible protein loading differences, the p-PKC-α/βII:total PKC-βII ratio was determined and found to be significantly greater in 17-week–old SHRs versus WKYs (Figure 1A). Similar results were obtained with the p-PKC-α/βII:total PKCα ratio (data not shown). After these initial experiments, we also examined PKC status at 14.5 weeks in a subset of SHRs and WKYs but found no change in p-PKC-α/βII at this age (data not shown). p-Akt^{Ser473} and the ratio of p-Akt^{Ser473}: total Akt were ≈70% greater in 10-week–old SHRs but unchanged in 17-week–old SHRs compared with age-matched WKYs (Figure 1B). GAPDH protein expression, used as a loading control, was similar among all of the groups (Figure 1B).

Table 1. Characteristics of WKYs and SHRs

Group	10-wk WKY	10-wk SHR	17-wk WKY	17-wk SHR
No.	6	6	6	6
Heart weight, mg	1015±54	1183±29*	1048±35	1297±43†
Body weight, g	245±5	297±5*	319±10	325±10†
Heart:body weight, mg:g	3.95±0.11	3.99±0.09	3.32±0.14	3.99±0.10†
Heart rate	398±10	410±8	422±14	480±18†
Caudal blood pressure, mm Hg				
Systolic	128±1	162±3*	138±2	184±7†
Diastolic	109±2	148±2*	114±3	159±5†
Mean arterial pressure	116±1	152±2*	122±2	167±4†

Data are mean±SE. WKY indicates normotensive control rats.
*$P<0.05$ vs 10-week–old WKYs.
†$P<0.05$ vs 17-week–old WKYs.

mTOR Signaling and Cardiovascular Variables in Rats Treated With Rapamycin

We reasoned that mTOR signaling might regulate the development of hypertrophy, because both Akt and S6 phosphorylation were greater at 10 but not 17 weeks in SHRs versus WKYs. To address this, 13-week–old SHRs were treated with rapamycin or vehicle for 3 weeks, a duration similar to those used in pressure-overload experiments.[20] In this experiment we did not use a rapamycin-treated WKY group because cardiac mass has been reported to be unaffected by this drug in control animals.[20,21] Thirteen-week–old SHRs were chosen because our preliminary studies indicated that cardiac hypertrophy is minimal at this time, and p-AKT is still elevated, whereas p-PKCα/βII is normal in 14.5-week–old SHRs. Inhibition of mTOR signaling by our rapamycin treatment regimen (Rap) was confirmed by ≈4-fold reduction in levels of p-S6$^{Ser235/236}$ and p-4E-BP1$^{Thr37/46}$ in SHR-Rap versus SHR-Veh and WKY-Veh (vehicle; Figure 2). p-Akt^{Ser473} was reduced ≈80% in SHR-Rap (Figure 2).

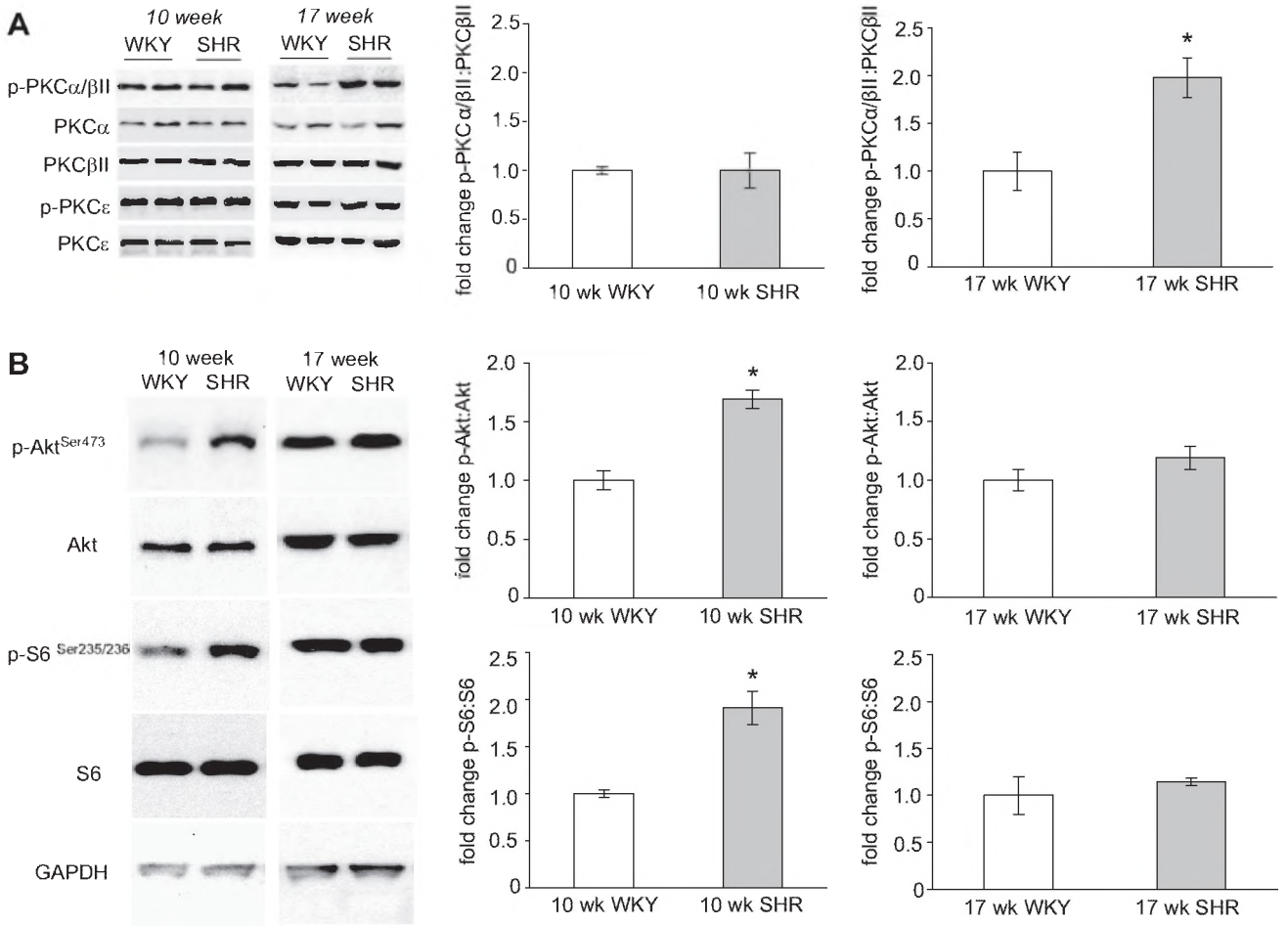

Figure 1. A, Western blot analysis of PKC-α, -βII, and -ε in hearts of 10-week–old SHRs during the development phase of cardiac hypertrophy and 17-week–old SHRs with established cardiac hypertrophy. Bar graphs represent fold change of p-PKC-α/βII:total PKC-βII in SHRs vs WKYs. B, Western blots of Akt and S6 in hearts of 10 week–old SHRs during the development phase of cardiac hypertrophy and 17-weeks-old SHRs with established cardiac hypertrophy. Bar graphs represent fold changes in p-Akt^{Ser473}:total Akt and p-S6:total S6 in SHRs vs age-matched WKYs. For all of the experiments, GAPDH was used as a loading control. For all of the bar graphs, data are presented as mean±SE; n=6 in all of the groups. *Significant difference at $P<0.05$.

Taken together, signaling through mTOR in the heart was markedly reduced in rapamycin-treated SHRs.

Heart weights were lower in SHR-Rap rats than SHR-Veh rats but still greater than WKY-Veh rats (Table 2). As expected, blood pressure was greater in SHRs versus WKYs, but surprisingly it was even higher in SHR-Rap rats versus SHR-Veh rats (Table 2). Rapamycin-treated SHRs had lower body weight than WKY-Veh or SHR-Veh rats (Table 2). Heart mass was, therefore, normalized to tibia length for the rapamycin studies. In spite of increased blood pressure in the SHR-Rap rats, cardiac hypertrophy was attenuated (heart: tibia length) versus SHR-Veh rats but was still greater than WKY-Veh rats (Figure 3A and 3B). Expression of atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP), markers of pathological cardiac hypertrophy and increased wall stress, was elevated in SHR-Veh versus WKY-Veh rats. Although hypertrophy was attenuated in SHR-Rap rats, the expression of ANP and BNP remained similar to SHR-Veh rats (Figure 3C).

It is possible that the combination of exaggerated hypertension and attenuated hypertrophy in SHR-Rap versus SHR-Veh rats might lead to increased wall stress and cardiac dysfunction. However, there was no echocardiographic evidence for LV dysfunction, because both ejection fraction and fractional shortening were similar in SHR-Veh and SHR-Rap rats (Figure 3D). Interventricular septal dimension, left ventricular diastolic dimension, and left ventricular posterior wall dimension were also similar in SHR-Rap versus SHR-Veh rats (data not shown).

Discussion

The contribution of mTOR signaling to cardiac hypertrophy that develops in response to a gradual increase in afterload, as occurs in the SHR, is unknown. In the present study, we observed activation of mTOR signaling in hearts of young SHRs during the developmental stage of cardiac hypertrophy. Pharmacologically inhibiting this pathway attenuated the extent of cardiac hypertrophy that ultimately occurs in this model. These are the first data indicating that mTOR signaling contributes importantly to cardiac hypertrophy in a clinically relevant model of hypertension.

Studies using pressure-overloaded mice and guinea pigs reported that P70 S6 ribosomal kinase phosphorylation is clearly correlated with cardiac hypertrophy[21,22] and that

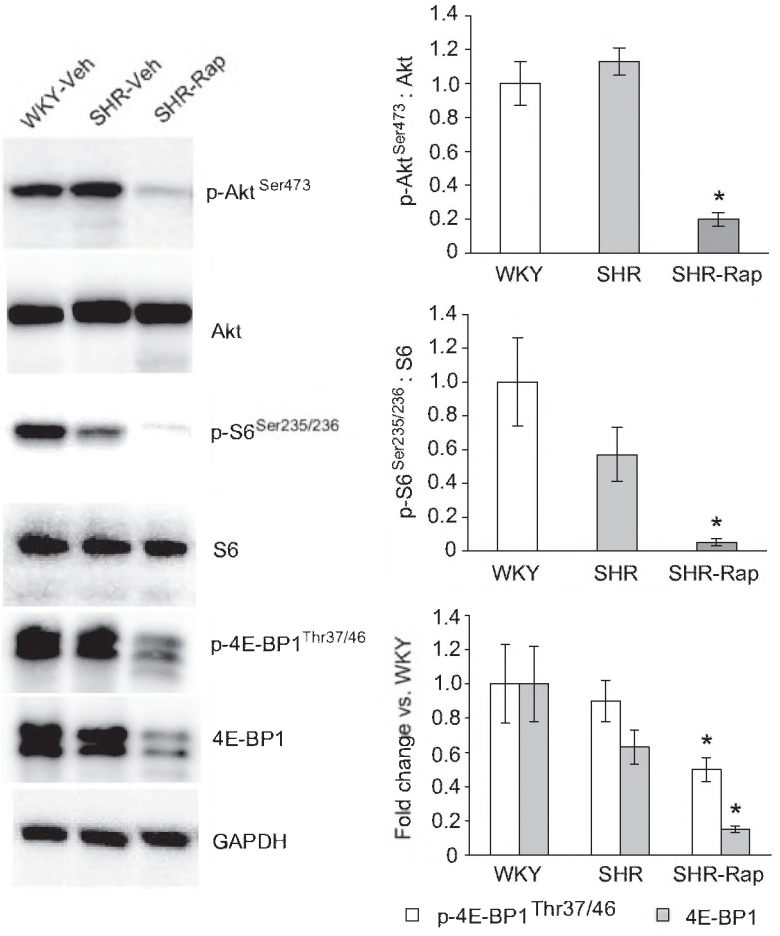

Figure 2. Impact of rapamycin treatment on mTOR-regulated signaling kinases. Bar graphs represent fold changes in p-Akt^{Ser473}:total Akt and p-S6:total S6. Bar graphs for 4E-BP1 represent fold change in p-4E-BP1 and total 4E-BP1 in SHRs vs age-matched WKYs. The ratio of p-4E-BP1:total 4E-BP1 is not shown because it does not convey the severe reduction in both 4E-BP1 phosphorylation and total 4E-BP1 protein that is evident from the blots themselves. For all of the experiments, GAPDH was used as a loading control. WKY indicates WKYs treated with saline vehicle; SHR, SHRs treated with saline vehicle; SHR-Rap, spontaneously hypertensive rats treated with rapamycin. n=7 WKY; n=6 SHR; n=9 SHR-Rap. *$P<0.05$ vs WKYs and SHRs.

cardiac-specific Akt overexpression increases activation of mTOR and results in cardiac hypertrophy.[9,10,23] The in vivo importance of mTOR has also been demonstrated in pressure-overloaded rodents where rapamycin treatment results in inhibition of mTOR, as determined by downstream effectors, such as P70 S6 ribosomal kinase, and attenuates cardiac hypertrophy evoked by aortic constriction.[21,24,25] The studies using pressure-overloaded models are important; however, it should be noted that aortic constriction creates local hypertension and does so in an abrupt manner. This process differs from the SHR that has gradually increasing systemic hypertension that eventually results in cardiac hypertrophy. The SHR may be considered to be more clinically relevant to the human experience, where uncontrolled hypertension leads to cardiac hypertrophy. In the present study, rapamycin-treated SHRs demonstrated a clear and robust reduction in the phosphorylation of S6 and 4E-BP1, both downstream targets of mTOR, and, thus, provided mechanistic evidence of the role of mTOR in the development of cardiac hypertrophy.

Table 2. Characteristics of WKYs and SHRs Treated With Vehicle or 2 mg/kg of Rapamycin

Variable	WKY-Veh	SHR-Veh	SHR-Rap
Age, wk	16	16	16
No.	7	11	14
Heart weight, mg	827±26	1244±21*	1080±18*†
Body weight, g	252±4	322±3*	269±3*†
Tibia length, mm	36.1±0.2	38.3±0.2*	38.0±0.2*
No.	7	6	9
Heart rate, bpm	376±11	390±10	405±13
Caudal blood pressure, mm Hg			
Systolic	139±6	189±7*	213±6*†
Diastolic	118±6	156±7*	171±6*
Mean arterial pressure	125±6	167±7*	184±5*†

Data are mean±SE. WKY indicates normotensive control rats treated with saline vehicle (Veh IP from 13 to 16 weeks of age). SHR-Rap rats were treated with 2 mg/kg of rapamycin from 13 to 16 weeks of age.

*$P<0.05$ vs WKYs.

†$P<0.05$ vs SHRs.

PKC isoforms have been reported to be mediators of cardiac function and hypertrophy. Overexpression or activation of PKC-βII, -ε, and -δ has been found to result in cardiac hypertrophy in mice.[26] Interestingly, mice with deletion of PKC-β still develop cardiac hypertrophy in response to pressure overload or phenylephrine,[27] and overexpression of PKC-α does not cause cardiac hypertrophy but results in diminished ventricular function.[28] Similarly, inhibition of conventional PKC isoforms (α, β, and γ) increases cardiac function in mice,[29] whereas adenoviral transfection of PKC-α reduces cardiac contractility in the normally hypercontractile PKC-α knockout mouse.[30] In contrast to our original hypothesis, we did not find activation of any PKC isoform in 10-week–old SHRs during the developmental phase of car-

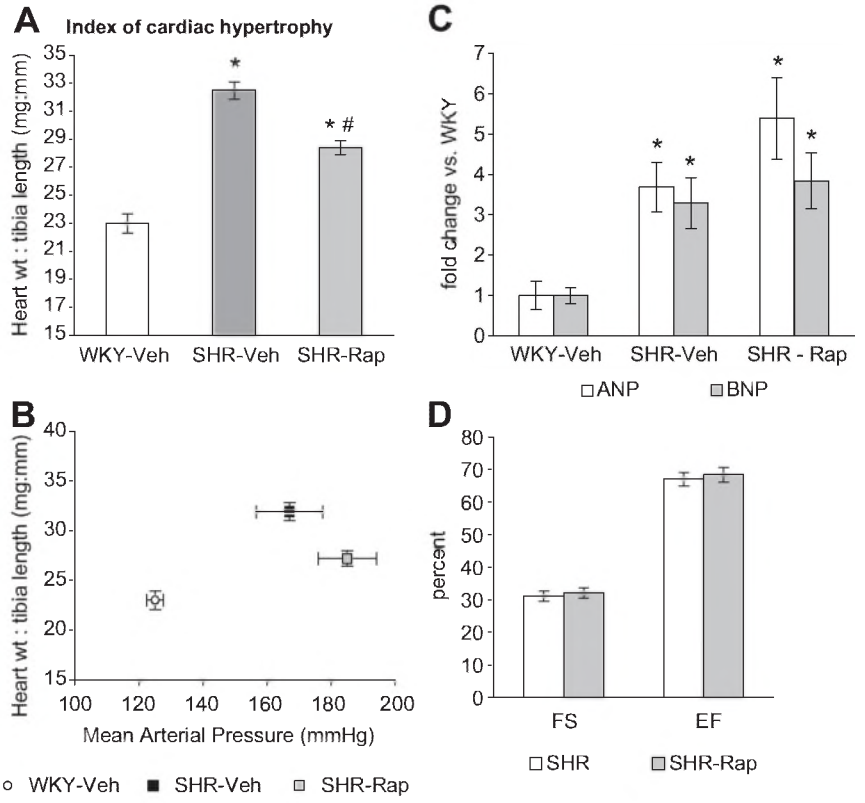

Figure 3. A, Index of cardiac hypertrophy expressed as heart weight:tibia length in rats treated with rapamycin at 2 mg/kg (Rap) or vehicle (saline) from 13 to 16 weeks of age (n=7 WKY; n=7 SHR; n=9 SHR-Rap). Heart:body weight was not used because of weight loss that occurred in SHRs after rapamycin treatment. B, Heart:tibia length plotted against mean arterial blood pressure (MAP) illustrates attenuation of cardiac hypertrophy in SHR-Rap rats in spite of significantly increased blood pressure compared with SHR-Veh rats (n=7 WKY; n=7 SHR; n=9 SHR-Rap). C, mRNA expression of ANP and BNP (n=7 WKY-Veh; n=7 SHR-Veh; n=9 SHR-Rap). D, In vivo assessment of cardiac function using parameters of percentage of fractional shortening (FS) and ejection fraction (EF; n=5 SHR; n=5 SHR-Rap). WKY indicates WKYs treated with saline vehicle; SHR, SHRs treated with saline vehicle; SHR-Rap, SHRs treated with rapamycin. *$P<0.05$ vs WKYs. †$P<0.05$ vs SHR-Veh rats.

diac hypertrophy. However, 17-week–old SHRs had an increase in p-PKC-α/βII. Although no other studies have examined PKC during the developmental phase of hypertrophy in the SHR, others have found PKC-α, -δ, and -ε activation in 6-month–old spontaneously hypertensive heart failure rats[31] and PKC-β activation in 16-week–old Dahl salt-sensitive rats.[32] Given the lack of PKC activation in hypertensive 10-week–old SHRs without hypertrophy, one may speculate that the PKC alterations in spontaneously hypertensive heart failure rats and Dahl salt-sensitive rats with established hypertrophy may be related to the regulation of cardiac function rather than growth.

In the present study, we found that treating SHRs with rapamycin for 3 weeks resulted in even greater blood pressure compared with vehicle-treated SHRs. This is consistent with previous studies reporting detrimental changes in kidney function, along with tubular atrophy and vascular pathology, after treating with 0.8 mg/g of rapamycin for 2 weeks.[33,34] Although hypertension has not been a reported adverse effect in human clinical trials using long-term rapamycin treatment for immunosuppression,[35] it should be noted that clinical use uses a lower dose of rapamycin compared with animal studies, such as the present one. Another observed adverse effect of rapamycin treatment was the weight loss that occurred in the SHR-Rap group. Although no other studies have used rapamycin to attenuate hypertrophy in genetically hypertensive models, several studies have used similar dosages of rapamycin in pressure-overloaded mice and rats. Studies using mice have not observed changes in body weight after either 1[21,25] or 4 weeks[20] of treatment with rapamycin, whereas pressure-overloaded rats show significant weight loss even after just 3 days of rapamycin.[24] To our knowledge, the present study is the first to treat SHRs with rapamycin.

It is seemingly contradictory that rapamycin treatment attenuated cardiac hypertrophy in SHRs in spite of greater blood pressure; however, this underscores the importance of mTOR signaling in stimulating cardiac growth during the developmental phase of hypertrophy. Given the persistence of this pathological stimulus (hypertension) in SHR-Rap rats, it is also not surprising that expression of ANP and BNP, both markers of pathological cardiac hypertrophy or increased wall stress, remained increased. Therefore, it appears that the reduction in hypertrophy after rapamycin treatment is attributable solely to the inhibition of mTOR, without fundamentally altering the pathological nature of the residual hypertrophy that develops in SHR-Rap rats or the increase in wall stress. Given the disproportional degree of hypertrophy versus hypertension in rapamycin-treated SHRs, we used echocardiography to evaluate ejection fraction and fractional shortening as measures of cardiac function. We have reported previously that 16-week–old SHRs with cardiac hypertrophy have normal cardiac function compared with age-matched WKYs.[36] In the present study, our data indicated that both ejection fraction and fractional shortening were also similar in vehicle and rapamycin-treated SHRs. We conclude that short-term rapamycin treatment does not adversely affect cardiac function; however, the effect of an extended (>3 weeks) period of rapamycin treatment on cardiac function remains unknown. A limitation, however, of the present study is the lack of histological analysis of hearts from rapamycin-treated and untreated SHRs. Histological analyses from previous studies indicate that myocyte size is increased in

14-week–old SHRs,[37] whereas cardiac fibrosis develops between 12 and 20 months of age.[38] However, in the present study, it is unknown to what degree rapamycin treatment may have altered cardiac structure with regard to myocyte size in 16-week–old SHRs.

Perspectives

Recent studies have reported that sirolimus (rapamycin) treatment can reduce LV hypertrophy in humans that is an adverse effect of kidney transplant[39] and heart transplant.[40,41] Not surprisingly, these reports have led to suggestions that rapamycin may have therapeutic potential for treating LV hypertrophy in cardiac transplant patients, as well as those with other etiologies, such as hypertension and myocardial infarction. However, our data indicate several factors that should be seriously considered in this regard. For example, transplant patients examined in previous studies did not have hypertension[40,41] or were under pharmacological blood pressure control,[39] whereas SHRs used in our study were severely hypertensive. This point is noteworthy, because our data indicate that rapamycin in the face of untreated hypertension may worsen the condition. Second, although we achieved more substantial reductions in cardiac hypertrophy in our study (≈50% reduction in developed hypertrophy) compared with those reported in the kidney or heart transplant patients (10% and ≈5% reduction in LV mass index, respectively), our rapamycin doses were much higher in SHRs versus patients (2 mg/kg IP per day in SHRs versus human total dose of 1 mg PO per day). Although our data suggest that a larger reduction of hypertrophy may be possible in clinical situations, safety and efficacy studies would be required to determine a dose of rapamycin that would strike a balance between optimal reductions in hypertrophy and reduced mortality without severe adverse effects. Finally, we have demonstrated in the SHR model that rapamycin markedly inhibits S6 and 4E-BP1; however, it is unclear whether this near total inhibition is actually required to attenuate cardiac hypertrophy. As such, the possibility exists that lower doses of rapamycin might be similarly efficacious in this regard. In light of this, we believe that further research is warranted to elucidate the degree of mTOR inhibition that is associated with LVH regression before any assumptions can be made as to the potential therapeutic use of rapamycin in patients with hypertrophy because of other causes, such as hypertension myocardial infarction.

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Disclosures

None.

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Online supplement

mTOR is a critical regulator of cardiac hypertrophy in Spontaneously Hypertensive Rats.

Will Soesanto[1], Han-yi Lin[1], Eric Hu[1], Shane Lefler[1], Sheldon E. Litwin[2], Sandra Sena[3], E. Dale Abel[3], J. David Symons[1], Thunder Jalili[1]

College of Health[1], Division of Cardiology[2], Human Molecular Biology and Genetics[3], University of Utah, Salt Lake City, UT 84112

METHODS

Animals

In the first series of experiments SHR were evaluated at time points when hypertension and cardiac growth were developing (10 weeks old, n=6) or established (17 weeks old, n=6), and results were compared to an identical number of age-matched WKY rats. Because we observed that mTOR signaling was increased in 10-week old rats, a second series of experiments was performed to test the hypothesis that mTOR is required for development of cardiac hypertrophy in these hypertensive animals. In this experiment thirteen-week old SHR were treated daily with rapamycin, an inhibitor or mTOR, (2 mg/kg i.p.; SHR-Rap, n=14) or saline (SHR-Veh, n=11) for 3 weeks, while age-matched WKY rats treated with saline served as controls (WKY-Veh, n=7). In these experiments we did not use a WKY rapamycin treated group because previous studies have shown that rapamycin does not affect cardiac mass in rodents that do not have pressure overload[1, 2]. To determine whether rapamycin compromised cardiac function in treated SHR cardiac function was evaluated in a subset of 16-week old SHR-Rap and SHR-Veh rats (n=5 per group).

Blood pressure and heart rate

Rats regained consciousness and were allowed to recover for 60-min. Arterial blood pressure and heart rate were measured over ~20 cardiac cycles (Biopac Systems Inc., Santa Barbara, CA). Next, rats were anesthetized again (5% isoflurane), the heart excised and placed immediately in iced physiological saline solution and trimmed of adherent tissue. After hearts were weighed, they were snap frozen in liquid nitrogen, and stored at -80°C. The left ventricle (LV) was used in subsequent experiments to examine signal transduction pathways.

RNA extraction and quantitative RT-PCR

After atrial natiuretic peptide (ANP) and brain natiuretic peptide (BNP) were expressed relative to cyclophilin (CPHN), values were normalized to the mean WKY value. Rat primer sequences were: CPHN-for 5'-AGCACTGGGGAGAAAGGATTTGG-3', CPHN-rev 5'-TCTTCTTGCTGGTCTTGCCATT-3', ANP-for 5'-GGGGGTAGGATTGACAGGAT-3', ANP-rev 5'-CTCCAGGAGGGTATTCACCA-3', BNP-for 5'-GACGGGCTGAGGTTGTTTTA-3', BNP-rev 5'-ACTGTGGCAAGTTTGTGCTG-3'.

Tissue homogenization and Western blotting

All homogenization procedures were performed at 4°C. The LV was homogenized with a tissuemizer in 1 ml of ice-cold RIPA buffer [50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% Nonidet P-40, 0.25% sodium deoxycholate, 1mM sodium orthovanadate, 1mM NaF, and 10 μl/mL Sigma protease inhibitor cocktail (Sigma, St. Louis, MO, cat. #P-8340)]. After homogenization, samples were sonicated on ice and centrifuged at 11,000 × g for 10 min at 4°C. Protein concentration was determined using a BioRad Protein assay (BioRad, Hercules,

CA) with bovine serum albumin (BSA) as a standard. Cardiac homogenates were stored at –80°C for subsequent Western blotting.

Antibodies directed against PKCα, βII, ε, δ, and p-PKCε^{Ser729} and GAPDH were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies directed against p-Akt^{Thr308}, p-Akt^{Ser473}, total Akt, p-PKCα/βII$^{Thr638/641}$ and p-PKCδ^{Thr505}, S6 ribosomal protein (S6), p-S6$^{ser235/236}$, eukaryotic translation initiation factor-4E binding protein-1 (4E-BP1), p-4E-BP1$^{Thr37/46}$ were purchased from Cell Signal Technology (Beverly, MA). All primary antibodies were incubated overnight at 4°C in a 1:1000 dilution in 5% BSA in Tris buffer with 0.05% Tween-20 (phospho specific antibodies) or 5% nonfat milk (non-phospho specific antibodies). Secondary antibody conjugated to horseradish peroxidase (goat anti-rabbit, Cell Signal Technology, Beverly, MA) was incubated for 1 h at 1:10,000 dilution. Signals were visualized by enhanced chemiluminescence (Cell Signal Technology, Beverly, MA). After exposure, membranes were stripped and probed with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a loading control (Chemicon, Temecula, CA). Relative band densities of immunoblots were measured using a Kodak GL1500 gel imaging system.

Myocardial Function

Rats were anesthetized with 2-5% isoflurane and two dimensional-guided M-mode images of the left ventricle were obtained using a General Electric Vivid 5 echocardiographic machine equipped with a 10-MHz transducer. Digital images were analyzed offline by a blinded observer.

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