FK506 Binding Protein 12/12.6 Depletion Increases Endothelial Nitric Oxide Synthase Threonine 495 Phosphorylation and Blood Pressure


Abstract—Chronic treatment with the immunosuppressive drug rapamycin leads to hypertension; however, the mechanisms are unknown. Rapamycin binds FK506 binding protein 12 and its related isoform 12.6 (FKBP12/12.6) and displaces them from intracellular Ca\(^{2+}\) release channels (ryanodine receptors) eliciting a Ca\(^{2+}\) leak from the endoplasmic/sarcoplasmic reticulum. We tested whether this Ca\(^{2+}\) leak promotes conventional protein kinase C–mediated endothelial NO synthase phosphorylation at Thr495, which reduces production of the vasodilator NO. Rapamycin treatment of control mice for 7 days, as well as genetic deletion of FKBP12.6, increased systolic arterial pressure significantly compared with controls. Untreated aortas from FKBP12.6\(^{-/-}\) mice and in vitro rapamycin-treated control aortas had similarly decreased endothelium-dependent relaxation responses and NO production and increased endothelial NO synthase Thr495 phosphorylation and protein kinase C activity. Inhibition of either conventional protein kinase C or ryanodine receptor restored endothelial NO synthase Thr495 phosphorylation and endothelial function to control levels. Rapamycin induced a small increase in basal intracellular Ca\(^{2+}\) levels in isolated endothelial cells, and rapamycin or FKBP12.6 gene deletion decreased acetylcholine-induced intracellular Ca\(^{2+}\) release, all of which were reversed by ryanodine. These data demonstrate that displacement of FKBP12/12.6 from ryanodine receptors induces an endothelial intracellular Ca\(^{2+}\) leak and increases conventional protein kinase C–mediated endothelial NO synthase Thr495 phosphorylation leading to decreased NO production and endothelial dysfunction. This molecular mechanism may, in part, explain rapamycin-induced hypertension. (Hypertension. 2007;49:569-576.)

Key Words: endothelium ■ hypertension ■ experimental ■ NO ■ NO synthase ■ protein kinases ■ vasorelaxation

Treatment with the immunosuppressive drug rapamycin decreases the incidence of organ rejection but causes hypertension in postorgan transplant patients.\(^{1,2}\) Rapamycin binds its intracellular target FK506 binding proteins 12 and 12.6 (FKBP12/12.6), and this complex inhibits the kinase mammalian target of rapamycin. A similar immunosuppressive drug, FK506, also binds FKBP12/12.6, but this complex inhibits the phosphatase calcineurin. Rapamycin and FK506 both produce hypertension,\(^{1,2}\) suggesting that a common mechanism leading to increased arterial pressure may be upstream of mammalian target of rapamycin or calcineurin. Xin et al\(^3\) genetically deleted FKBP12.6 in mice and reported elevated blood pressures; however, the mechanisms by which alterations in FKBP12/12.6 might affect blood pressure regulation have not been examined.

In addition to binding rapamycin and FK506, the immunophilins FKBP12 and FKBP12.6 bind to intracellular Ca\(^{2+}\) release channels (ryanodine receptors; RyRs). Most cells have a higher concentration of FKBP12 than FKBP12.6, but FKBP12.6 has a 100-fold higher affinity for RyR2, the predominant isoform in vascular tissue, compared with FKBP12.\(^{4,6}\) FKBP12/12.6 stabilizes a closed state of RyR, and displacement from the channel by rapamycin or FK506 creates a Ca\(^{2+}\) leak by increasing the probability and duration of RyR opening.\(^{6,7}\)

Endothelium-derived NO plays a major role in vascular tone and blood pressure regulation.\(^8\) FK506 treatment of rats decreases vascular NO production; however, the mechanisms by which rapamycin, FK506, or FKBP12.6 gene deletion affect endothelial NO biosynthesis are unknown. The formation of NO from L-arginine via the enzymatic activity of endothelial NO synthase (eNOS) depends on Ca\(^{2+}\)/calmodulin and is regulated in part by its phosphorylation status.\(^10\) Protein kinase C (PKC) phosphorylates eNOS at an inhibitory site, threonine 495 (Thr495), in vitro and is activated by a lower concentration of intracellular Ca\(^{2+}\) (EC\(_{50}\) = 200 \(\mu\)mol/L) than eNOS (EC\(_{50}\) = 400 \(\mu\)mol/L).\(^{11,12}\) Therefore, PKC is the most likely kinase for coupling minor changes in cytoplasmic Ca\(^{2+}\) to decreased eNOS activity, leading to endothelial dysfunction and hypertension.

The roles of FKBP12/12.6 in endothelial function and blood pressure regulation are unknown. We hypothesized that...
FKBP12/12.6 depletion from intracellular Ca\(^{2+}\) release channels via rapamycin or genetic deletion causes a Ca\(^{2+}\) leak, which increases PKC-mediated phosphorylation of eNOS at Thr495 and decreases NO production and endothelium-dependent dilation. To test this, we measured blood pressures in FKBP12.6\(^{-/-}\) mice and in control mice treated for 7 days with rapamycin. We also measured endothelium-dependent dilation, NO production, eNOS phosphorylation, and PKC activity in isolated aortas from FKBP12.6\(^{-/-}\) mice and in control aortas acutely treated in vitro with rapamycin to examine the direct vascular effects. In addition, we measured intracellular Ca\(^{2+}\) levels in rapamycin-treated and nontreated aortic endothelial cells from FKBP12.6\(^{-/-}\) and control mice. Rapamycin or genetic deletion of FKBP12.6 produced hypertension, as well as an endothelial intracellular Ca\(^{2+}\) leak, increased conventional PKC (cPKC)-mediated phosphorylation of eNOS at Thr495, and reduced NO production and endothelium-dependent dilation.

**Methods**

**Animals and Blood Pressure Measurements**

Male C57Bl/6 (Harlan, Indianapolis, IN), FKBP12.6\(^{-/-}\) (Pitzer, New London, CT), and FKBP12.6\(^{+/+}\) mice aged 10 to 18 weeks were used in all of the experiments. FKBP12.6 mice were genotyped using tail DNA and the following primers (5\’ to 3\’): mutant forward-TGGGCGGAATGGGCTGAC; mutant reverse- TGGGCGAC- TCGGGCACAC; wild-type forward- AAGCAGTCCCTGGAATATA; and wild-type reverse-CGGGCGGGAATGATGAGATAA. All of the procedures were approved by the Baylor College of Medicine Institutional Animal Care and Use Committee. Tail-cuff systolic blood pressures (IITC, Inc) were measured at baseline and after 7 days of treatment with rapamycin (2 mg/kg per day, IP) or DMSO (0.2% final concentration). We determined previously that there were no differences in blood pressure, endothelium-dependent dilation, or NO production between C57Bl/6 and FKBP12.6\(^{+/+}\) mice; therefore, aortas from these mice were grouped together and are collectively referred to as controls.

**Organ Chamber Experiments**

Mice were anesthetized with isoflurane and euthanized by cervical dislocation. The thoracic aorta was immediately excised and placed in cold physiological salt solution containing: 119.0 mmol/L of NaCl, 4.7 mmol/L of KCl, 1.18 mmol/L of KH\(_2\)PO\(_4\), 1.17 mmol/L of MgSO\(_4\)-7H\(_2\)O, 25 mmol/L of NaHCO\(_3\), 11.1 mmol/L of dextrose, and 2.5 mmol/L of CaCl\(_2\). Isolated endothelium-intact aortic rings (3 to 4 mm) were connected to an isometric force transducer in a custom-made 15-mL organ chamber filled with 37°C physiological salt solution and bubbled with 95% O\(_2\)-5% CO\(_2\). Isolated endothelium-intact aortic rings (3 to 4 mm) were connected to an isometric force transducer in a custom-made 15-mL organ chamber filled with 37°C physiological salt solution and bubbled with 95% O\(_2\)-5% CO\(_2\). All of the experiments were performed in the presence of indomethacin (10 \(\mu\)mol/L) to inhibit cyclooxygenase and to examine NO-vascular reactivity. To examine the direct effects of rapamycin on endothelium-dependent relaxation responses, aortic rings were incubated with rapamycin (1 \(\mu\)mol/L) for 20 minutes. Concentration-force curves were obtained in a half-log, cumulative fashion to acetylcholine (ACH) and sodium nitroprusside after contraction to an EC\(_{50}\) concentration of phenylephrine (1 \(\mu\)mol/L). Relaxation responses were also assessed after an NO synthase inhibitor N\(^\circ\)-nitro-l-arginine (l-NNA; 10 \(\mu\)mol/L, 20 minutes), a general PKC inhibitor chelerythrine (1 \(\mu\)mol/L, 20 minutes), a cPKC-specific inhibitor Go\_6976 (1 \(\mu\)mol/L, 20 minutes), a RyR inhibitor ryanodine (50 \(\mu\)mol/L, 60 minutes), superoxide dismutase (150 U/mL, 30 minutes), a reduced nicotinamide-adenine dinucleotide phosphate oxidase inhibitor apocynin (10 \(\mu\)mol/L, 60 minutes), or the tetrahydrobiopterin precursor sepiapterin (100 \(\mu\)mol/L, 30 minutes).

**Preparation of Vascular Homogenates**

Endothelium-intact aortic rings were incubated in the absence and presence of rapamycin (1 \(\mu\)mol/L, 20 minutes), G66976 (1 \(\mu\)mol/L, 20 minutes), and/or ryanodine (50 \(\mu\)mol/L, 60 minutes). The rings were homogenized in the presence of fresh protease and phosphatase inhibitors. The homogenate was centrifuged at 11 000 rpm for 10 minutes at 4°C. Protein concentration was determined by Lowry assay using bovine serum albumin as the standard.

**NO Production**

An assay using the cell-permeable dye 4-amino-5-methylamino-2,7\'-difluorofluorescein diacetate (DAF-FM diacetate; Invitrogen Molecular Probes), described previously,\(^{14}\) was modified for vascular tissue. In brief, 20 \(\mu\)g of protein, 12 \(\mu\)mol/L of DAF-FM diacetate, and water were added to the assay buffer to a final volume of 1 mL, and the sample was stirred continuously and warmed to 37°C. Fluorescence was recorded for 20 minutes using a spectrofluorometer with an excitation wavelength of 510 nm, an emission wavelength of 530 nm, a bandwidth of 4 nm, and a count rate of 1 per second. Fluorescence was also measured after NOS inhibition with l-NNA (100 \(\mu\)mol/L, 20 minutes). NO production was determined by measuring peak fluorescent counts in the absence of l-NNA minus peak fluorescent counts in the presence of l-NNA.

**Immunoblotting**

Vascular homogenates were separated by electrophoresis on 7.5%-Laemmli SDS polyacrylamide gels and then transferred to Immobilon-FL PVDF membranes (Millipore) overnight at 4°C. Western blot analyses were performed using the following primary antibodies: eNOS 1:2500 (BD Biosciences Transduction Laboratories), phospho-eNOS Thr495 1:1000 (Upstate), phospho-eNOS serine (Ser) 1177 1:1000 (Cell Signaling), and phospho-PKC Ser660 1:1000 (Cell Signaling). PKC requires phosphorylation at Ser660 for activation and has been used as a measure of PKC activity in vascular tissue previously.\(^{15}\) Secondary antibodies consisted of anti-mouse and anti-rabbit IgGs conjugated to Alexa-Fluor 680 (Invitrogen Molecular Probes) and IR800Dye (Rockland Immunochemicals), respectively. The bands were identified using infrared visualization (Odyssey System, LI-COR Biosciences), and densitometry was performed using the Odyssey software. Coolmassie blue staining was performed on the membranes, and densitometry of a band with a molecular weight of 45 kDa on the PVDF membrane was analyzed using a Bio-Rad Fluor2-Multimager and Bio-Rad Quantity One software (v 4.5.0).

**Isolation of Mouse Aortic Endothelial Cells**

Primary mouse aortic endothelial cells (MAECs) were isolated using Matrigel as described previously.\(^{16}\) Aortic rings were placed in mouse endothelial cell medium on a glass coverslip coated with Matrigel (BD Biosciences Discovery Labware) for 3 to 4 days. Aortic rings were removed after endothelial cell outgrowth was observed. Primary endothelial cells were used for all of the experiments.

**Intracellular Ca\(^{2+}\) Imaging**

MAECs were loaded with Fura-2 AM (10 \(\mu\)mol/L) diluted in physiological salt solution and were imaged on a Nikon Eclipse E600FN equipped with a Cascade 512B charge-coupled device camera (Photometrics). Cells were placed in 0-Ca\(^{2+}\) physiological salt solution (no CaCl\(_2\), 100 \(\mu\)mol/L EGTA) and alternately excited at 340 and 380 nm. The ratio of 340/380 nm was continuously monitored for >10 endothelial cells. After a 5-minute baseline, some cells were treated with rapamycin (1 \(\mu\)mol/L) for 15 minutes. To increase Ca\(^{2+}\) mobilization from intracellular stores, cells were treated with ACh (1 \(\mu\)mol/L) and monitored for 5 minutes. Sixteen-bit time-lapsed fluorescent images (8±30 Hz) were collected at an emission wavelength of 510 nm throughout. In parallel experiments, endothelial cells were pretreated with ryanodine (50 \(\mu\)mol/L, 60 minutes) to inhibit RyR opening.
that reported by Xin et al (1452 mm Hg).3

Results

Effect of Rapamycin and FKBP12.6 Gene Deletion on Systolic Blood Pressure

Rapamycin treatment for 7 days increased systolic arterial pressure from 113±3 mm Hg to 134±3 mm Hg (P<0.05 versus controls; Figure 1), whereas arterial pressure did not change in vehicle-treated controls (baseline: 113±4 mm Hg; 7-day vehicle: 111±4 mm Hg). Systolic blood pressure was 142±2 mm Hg in FKBP12.6−/− mice (Figure 1), which was also significantly higher than controls and was comparable to that reported by Xin et al (145±2 mm Hg).3

Effect of Rapamycin and FKBP12.6 Gene Deletion on Endothelium-Dependent and -Independent Relaxation

Maximal relaxation responses to ACh were decreased significantly in rapamycin-treated control aortas compared with nontreated controls (relaxation from phenylephrine-induced contraction: rapamycin=29±10% and controls=83±4%; P<0.05 versus controls; Figure 2). Maximal relaxation responses were similarly decreased in aortas from FKBP12.6−/− mice (relaxation from phenylephrine-induced contraction: FKBP12.6−/−=23±10%; P<0.05 versus controls; Figure 2). NO synthase inhibition with l-NNA (10 μmol/L) abolished relaxation responses and caused a constriction to ACh in all of the groups (Figure 2). Inhibition of PKC with chelerythrine (10 μmol/L, 20 minutes) restored relaxation responses to ACh in rapamycin-treated control aortas and aortas from FKBP12.6−/− mice and had no effect on relaxation responses in untreated control aortas (data not shown). Specific inhibition of cPKC with Go6976 (1 μmol/L) increased ACh-mediated relaxation responses in rapamycin-treated control aortas and aortas from FKBP12.6−/− mice beyond that of controls (Figure 3A). Go6976 (1 μmol/L) also significantly increased ACh-mediated relaxation responses in untreated control aortas (Figure 3A). Inhibition of RyR opening with ryanodine (50 μmol/L) also restored relaxation responses in rapamycin-treated control aortas and aortas from FKBP12.6−/− mice (Figure 3B) but had no significant effect on relaxation responses in untreated control aortas. The improvements in ACh-induced relaxation after chelerythrine, Go6976, or ryanodine were blocked by l-NNA (data not shown). Superoxide dismutase (150 U/mL), apocynin (10 μmol/L), or sepiapterin (100 μmol/L) had no significant effect on ACh-mediated relaxation responses in untreated or rapamycin-treated control aortas or in aortas from FKBP12.6−/− mice (data not shown), suggesting that FKBP12/12.6 depletion did not increase superoxide production or uncouple eNOS.

Endothelium-independent relaxation responses to sodium nitroprusside were not different in rapamycin-treated control aortas or aortas from FKBP12.6−/− mice compared with controls (data not shown). Thus, vascular smooth muscle relaxation in response to an NO donor was not altered by rapamycin or by genetic deletion of FKBP12.6.

Effect of Rapamycin and FKBP12.6 Gene Deletion on NO Production

Using an assay that measures NO production in vascular homogenates, we found that acute in vitro rapamycin treatment of control aortas decreased peak NO production ≈80% compared with untreated controls (l-NNA–sensitive peak DAF-FM fluorescent counts: rapamycin=71 951±27 198 and controls=345 764±56 478; P<0.05 versus controls; Figure 4). Similarly, genetic deletion of FKBP12.6 decreased...
Because PKC is known to inhibit NO production, we verified that the improvement in relaxation responses induced by the cPKC-specific inhibitor G06976 was because of increased NO production. Isolated control aortas treated with rapamycin and nontreated aortas from FKBPI2.6−/− mice were incubated with G06976 before homogenization. G06976 restored NO production in both groups (l-NNA–sensitive peak DAF-FM fluorescent counts: rapamycin + G06976 = 460 821 ± 49 878 and FKBPI2.6−/− + G06976 = 414 578 ± 24 125; P>0.05 versus controls; Figure 4). G06976 slightly increased NO production in control aortas; however, this did not reach statistical significance (Figure 4). We also tested whether the restoration of endothelium-dependent dilation by ryanodine was associated with increased NO production. Inhibition of the RyR opening restored NO production in control aortas treated with rapamycin and nontreated aortas from FKBPI2.6−/− mice to control levels (Figure 4).

**Effect of Rapamycin and FKBPI2.6 Gene Deletion on eNOS and Phospho-eNOS Thr495 Expression**

eNOS protein levels were not different in rapamycin-treated control aortas or in aortas from FKBPI2.6−/− mice compared with untreated controls (Figure 5). However, rapamycin or FKBPI2.6 gene deletion increased phosphorylation of eNOS at Thr495 (Figure 5). We hypothesized that an intracellular Ca2+ leak activates cPKC leading to eNOS Thr495 phosphorylation; therefore, we incubated rapamycin-treated control aortas and aortas from FKBPI2.6−/− mice with the cPKC inhibitor G06976 or the RyR inhibitor ryanodine and measured eNOS Thr495 phosphorylation. Both G06976 and ryanodine reversed the increase in eNOS Thr495 phosphorylation by rapamycin or genetic deletion of FKBPI2.6 (Figure 5). G06976 also decreased eNOS Thr495 phosphorylation in untreated control aortas (Figure 5). These data support and extend previous findings that PKC and, more specifically, cPKC isoforms phosphorylate eNOS at Thr495 and decrease NO production.

We also analyzed whether the increase in eNOS Thr495 phosphorylation induced by acute in vitro rapamycin treatment or FKBPI2.6 gene deletion was associated with altered eNOS Ser1177 phosphorylation. eNOS phosphorylation at Ser1177 stimulates NO production, and this occurs after dephosphorylation of Thr495.12 eNOS Ser1177 phosphorylation was abolished in control aortas acutely treated with rapamycin and in aortas from FKBPI2.6−/− mice (Figure I, available online at http://hyper.ahajournals.org). cPKC or RyR inhibition restored eNOS Ser1177 phosphorylation in these vessels and tended to increase eNOS Ser1177 phosphorylation in untreated control aortas (P=0.089 for G06976). These data support the findings of Michell et al.,12 who found that removal or inhibition of eNOS phosphorylation at Thr495 increases eNOS phosphorylation at Ser1177 and NO production.

**Effect of Rapamycin and FKBPI2.6 Gene Deletion on PKC Activity**

Acute rapamycin treatment of control aortas increased PKC activity compared with controls as determined by PKC

---

**Figure 3.** Inhibition of cPKC or ryanodine receptor opening restored endothelium-dependent relaxation responses after acute in vitro rapamycin (1 μmol/L) or FKBPI2.6 gene deletion. Effects of (A) the cPKC-specific inhibitor G06976 or (B) ryanodine (Ryan) on relaxation responses to acetylcholine in rapamycin-treated control aortas (Rapa) and aortas from FKBPI2.6−/− mice (12.6−/−). Results are expressed as mean±SEM and parentheses contain the number of mice. *P<0.05 vs control.

**Figure 4.** Rapamycin or FKBPI2.6 gene deletion decreased NO production, which was reversed by cPKC or RyR inhibition. Effects of acute in vitro rapamycin (Rapa) treatment (1 μmol/L) or FKBPI2.6 gene deletion (12.6−/−), with and without cPKC inhibition (G06976) or RyR inhibition (ryanodine), on vascular NO production as assessed by peak l-NNA–sensitive DAF-FM fluorescence. Results are expressed as mean±SEM (n>3 mice for each group). *P<0.05 vs control.

**Figure 5.** Effect of Rapamycin and FKBPI2.6 Gene Deletion on PKC Activity. Acute rapamycin treatment of control aortas increased PKC activity compared with controls as determined by PKC.
Ser660 phosphorylation (Figure 6). Untreated aortas isolated from FKBP12.6/H11002 mice also exhibited an increase in PKC Ser660 phosphorylation compared with controls (Figure 6). Ryanodine significantly decreased PKC Ser660 phosphorylation in rapamycin-treated control aortas and nontreated aortas from FKBP12.6/H11002 mice (Figure 6), suggesting that a RyR-mediated calcium leak leads to activation of cPKC and phosphorylation of eNOS at Thr495.

**Effect of Rapamycin and FKBP12.6 Gene Deletion on Intracellular Ca^{2+} Levels**

To examine whether a Ca^{2+} leak induced by the displacement of FKBP12/12.6 from RyRs, known to occur in myocytes,6,7 also occurs in endothelial cells, we measured endothelial intracellular Ca^{2+} release after pharmacological or genetic depletion of FKBP12/12.6. In primary MAECs from control mice, rapamycin caused a Ca^{2+} leak of ≈10% to 20% of the maximal ACh-induced Ca^{2+} release in nontreated control MAECs (Figure 7A). In response to ACh, rapamycin-treated MAECs had a markedly decreased peak Ca^{2+} release of 27±7% (P<0.05 versus controls) of controls followed by a sustained Ca^{2+} release of 16% to 18% (Figure 7C).

Because FKBP12/12.6 binds to RyR and stabilizes a closed state of the channel,6,7 we tested whether inhibition of RyR with ryanodine could block the rapamycin-induced Ca^{2+} leak. In MAECs from control mice, ryanodine abolished the intracellular Ca^{2+} leak after rapamycin (Figure 7A). Furthermore, ryanodine restored ACh-induced Ca^{2+} release in FKBP12.6/−/ mice to 80±10% of controls (Figure 7C). In the presence of ryanodine, rapamycin augmented ACh-induced Ca^{2+} release in MAECs from control and FKBP12.6/−/ mice (Figure 7B and 7C).

**Discussion**

The mechanisms by which rapamycin treatment causes hypertension are unknown. The present study demonstrates that rapamycin decreases endothelium-dependent dilation and NO production, which may occur because of an intracellular Ca^{2+} leak leading to cPKC-mediated phosphorylation of eNOS at Thr495. Furthermore, the detrimental effects of rapamycin appear to be mediated by displacement of FKBP12/12.6 from intracellular Ca^{2+} release channels, because mice with a genetic deletion of FKBP12.6 demonstrate similarly altered intracellular Ca^{2+} stores, PKC activity, eNOS Thr495 phosphorylation, NO production, endothelium-dependent dilation, and systolic arterial pressures.
FKBP12/12.6 Depletion Increases Blood Pressure and Endothelial Dysfunction

The role of FKBP12/12.6 in endothelial function has not been examined previously in the absence of rapamycin. In addition, it was unknown whether rapamycin increased blood pressure by direct or indirect vascular effects. Xin et al. reported cardiac hypertrophy and hypertension in male FKBP12.6/H11002/H11002 mice; however, they did not examine how genetic deletion of FKBP12.6 elevated blood pressure. Here we demonstrate that rapamycin, which binds FKBP12/12.6 and displaces the immunophilins from RyRs, or genetic deletion of FKBP12.6 caused hypertension. To delineate the effects of elevated blood pressure versus the direct vascular effects of rapamycin on endothelial function, we treated control aortas with rapamycin in vitro and examined endothelium-dependent dilation and NO production. Acute rapamycin treatment decreased both of these measures in control aortas. Aortas from FKBP12.6/H11002/H11002 mice also exhibited decreased endothelium-dependent dilation and NO production, which may explain why these mice develop hypertension and cardiac hypertrophy. The FKBP12/12.6-binding drug FK506 has also been shown to attenuate eNOS activity and relaxation responses in rat aortas. Taken together, these findings suggest that depletion (pharmacological-induced displacement or genetic deletion) of FKBP12/12.6 decreases vascular NO production and increases blood pressure.

FKBP12/12.6 Depletion Alters eNOS Phosphorylation

The attenuation of endothelial NO biosynthesis and increase in blood pressure by rapamycin or FKBP12.6 gene deletion did not appear to be mediated by increased superoxide production or uncoupled eNOS but rather correlates strongly with an increase in eNOS Thr495 phosphorylation. In vitro studies have shown that phosphorylation of eNOS at Thr495, located in the calmodulin binding domain of eNOS, reduces calmodulin binding and, thus, eNOS activation. Functional evidence to support this comes from a study in which Thr495 was mutated to an aspartate (phosphomimetic).18 The phosphomimetic eNOS bound almost no calmodulin, and eNOS activity was abolished, except in the presence of supraphysiological concentrations of Ca2+ and calmodulin.18 The absence of relaxation responses to low concentrations of ACh but relaxation responses to high concentrations of ACh in control vessels acutely treated with rapamycin and vessels from FKBP12.6/H11002/H11002 mice (Figure 2) may provide ex vivo support of these findings. Our data support an association between increased eNOS Thr495 phosphorylation and hypertension.

In addition to an increase in eNOS phosphorylation at an inhibitory site, we also found that acute in vitro rapamycin treatment or FKBP12.6 gene deletion lead to a decrease in eNOS phosphorylation at a stimulatory site, Ser1177. Al-
FKBP12/12.6 Depletion Increases PKC Activity
FKBP12/12.6 gene deletion (12.6−/−) on endothelial intracellular Ca2+ levels. A, Rapamycin (1 μmol/L) caused an intracellular Ca2+ leak in aortic endothelial cells from control mice, which was prevented by ryanodine (Ryan; 50 μmol/L). B, ACh-induced intracellular Ca2+ release (1 μmol/L) was markedly decreased in rapamycin-treated control aortic endothelial cells but augmented after ryanodine. C, ACh-induced intracellular Ca2+ release (1 μmol/L) was also decreased in aortic endothelial cells from FKBP12.6−/− mice but restored to control levels after ryanodine and augmented after rapamycin and ryanodine. All experiments were performed in the absence of extracellular Ca2+. Results are expressed as the mean increase in 340/380 nm ratio as a percentage of the peak response to ACh in untreated controls (n=10 cells from >3 mice for each group).

FKBP12/12.6 Depletion Increases PKC Activity
PKC has been shown recently to phosphorylate eNOS Thr495 in vitro, but an association between PKC and NO production has been known for many years. Phorbol ester, a PKC agonist, decreases NO-mediated cGMP production, reduces eNOS activity ~50%, and interferes with the activation of eNOS by Ca2+/calmodulin.20–24 In 1995, Hirata et al25 reported that PKC directly phosphorylates eNOS, and downregulation of PKC increased agonist-induced NO release 2-fold. Fleming et al18 reported that PKC inhibition or downregulation decreased eNOS Thr495 phosphorylation and potentiated the production of cGMP. In the current study, rapamycin or FKBP12.6 gene deletion increased PKC activity and eNOS Thr495 phosphorylation. Inhibition of cPKC with Gö6976 decreased eNOS Thr495 phosphorylation and increased eNOS Ser1177 phosphorylation, NO production, and endothelium-dependent dilation in these vessels. Gö6976 also decreased eNOS Thr495 phosphorylation, tended to increase eNOS Ser1177 phosphorylation and NO production, and increased endothelium-dependent dilation in control aortas, suggesting that, at rest, cPKC may exert a small inhibitory effect on eNOS activity. Two other molecules that lead to endothelial dysfunction, homocysteine and β-amyloid peptides, have also been shown to activate PKC leading to increased eNOS Thr495 phosphorylation and reduced NO production, which supports our findings.15,16

FKBP12/12.6 Depletion Induces an Intracellular Ca2+ Leak in Endothelial Cells
Because PKC can phosphorylate eNOS Thr495 leading to decreased calmodulin binding, eNOS activity, and NO production, alterations in intracellular Ca2+ and PKC activation may have marked effects on endothelium-dependent dilation and blood pressure. PKC is activated at a lower concentration of intracellular Ca2+ than eNOS, suggesting that the magnitude and localization of intracellular Ca2+ release play a major role in the activation of these enzymes. In the absence of extracellular Ca2+, we found that acute treatment of isolated endothelial cells with rapamycin produced an intracellular Ca2+ leak and decreased ACh-induced intracellular Ca2+ release. We were not able to observe a Ca2+ leak in endothelial cells from FKBP12.6−/− mice; however, this leak was most likely compensated for by increased Ca2+ extrusion. This would result in a decrease in intracellular Ca2+ stores and most likely explains the decreased ACh-induced intracellular Ca2+ release in MAECs from FKBP12.6−/− mice.

The role of RyRs in endothelial cell function is not well defined. We found that inhibition of RyR not only prevented a rapamycin-induced endothelial cell Ca2+ leak, known to occur in other cell types, such as skeletal and cardiac myocytes, but also attenuated PKC activation, decreased eNOS Thr495 phosphorylation, and increased eNOS Ser1177 phosphorylation, NO production, and endothelium-dependent dilation after acute in vitro rapamycin treatment or FKBP12.6 gene deletion. These findings suggest that Ca2+ leaks from RyR may preferentially activate cPKC and alter eNOS phosphorylation. Whether RyRs and inositol triphosphate receptors control the same or distinct endoplasmic Ca2+ stores is controversial; however, our data suggest significant over-
lack, because inhibition of RyR opening in aortic endothelial cells from FKBPI2.6−/− mice restored intracellular Ca2+ release induced by ACh, which releases Ca2+ via ryanodine- and inositol triphosphate–sensitive stores. Furthermore, Ca2+ release from RyRs appears to reduce intracellular Ca2+ stores, because ryanodine unmasked a rapamycin-induced augmentation in ACh-induced Ca2+ release. More studies are needed to clarify the contribution of RyRs to immunosuppressive drug-induced hypertension.

In conclusion, FKBPI2/12.6 contributes to endothelial function and blood pressure regulation by stabilizing endothelial intracellular calcium release channels. Rapamycin-induced displacement of FKBPI2/12.6 from RyRs or genetic deletion of FKBPI2.6 elevates blood pressure, which may be mediated by an endothelial intracellular Ca2+ leak leading to cPKC-mediated phosphorylation of eNOS at Thr495 and endothelial dysfunction.

**Perspectives**

This study elucidated a molecular mechanism that potentially contributes to rapamycin-induced hypertension. If rapamycin suppresses the immune system by inhibiting mammalian target of rapamycin, then the development of direct mammalian target of rapamycin inhibitors that do not involve FKBPI2/12.6 may reduce the detrimental cardiovascular effects associated with rapamycin.

**Sources of Funding**

This work was supported by grants AR41802 and AR050503 (S.L.H.) from the National Institutes of Health and an American Heart Association Scientist Development Grant (B.M.M.).

**Disclosures**

None.

**References**


FK506 Binding Protein 12/12.6 Depletion Increases Endothelial Nitric Oxide Synthase Threonine 495 Phosphorylation and Blood Pressure

Cheng Long, Leslie G. Cook, Susan L. Hamilton, Gang-Yi Wu and Brett M. Mitchell

*Hypertension*. 2007;49:569-576; originally published online January 29, 2007;
doi: 10.1161/01.HYP.0000257914.80918.72

*Hypertension* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2007 American Heart Association, Inc. All rights reserved.
Print ISSN: 0194-911X. Online ISSN: 1524-4563

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://hyper.ahajournals.org/content/49/3/569

Data Supplement (unedited) at:
http://hyper.ahajournals.org/content/suppl/2007/02/01/01.HYP.0000257914.80918.72.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in *Hypertension* can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to *Hypertension* is online at:
http://hyper.ahajournals.org/subscriptions/
ONLINE SUPPLEMENT FOR:

FKBP12/12.6 DEPLETION INCREASES ENDOTHELIAL NITRIC OXIDE SYNTHASE
THREONINE 495 PHOSPHORYLATION AND BLOOD PRESSURE

Cheng Long, PhD, Leslie G. Cook, BS, Susan L. Hamilton, PhD, Gang-Yi Wu, PhD, and
Brett M. Mitchell, PhD

Department of Molecular Physiology and Biophysics
Baylor College of Medicine
One Baylor Plaza
Houston, TX 77030

Short Title – FKBP12/12.6 and eNOS Phosphorylation

Correspondence to:
Brett M. Mitchell, PhD
Dept. of Molecular Physiology and Biophysics
Baylor College of Medicine
One Baylor Plaza
Houston, TX 77030
Phone: 713.798.3616   Fax: 713.798.3475   Email: brettm@bcm.tmc.edu
FIGURE LEGEND

Supplemental Figure I. Rapamycin or FKBP12.6 gene deletion decreased eNOS Ser1177 phosphorylation which was reversed by inhibition of cPKC or ryanodine receptor opening. Effects of acute \textit{in vitro} rapamycin (Rapa) treatment (1 \(\mu\)mol/L) or FKBP12.6 gene deletion (12.6 -/-), with and without Gö6976 or ryanodine, on eNOS Ser1177 phosphorylation. A, Representative Western blots showing aortic eNOS Ser1177 phosphorylation and eNOS expression. B, Densitometry for ratio of eNOS Ser1177 phosphorylation to eNOS expression. Results are expressed as mean \(\pm\) SEM (n>3 mice for each group). *p<0.05 vs control.
Figure I

A

Phospho-eNOS-1177

<table>
<thead>
<tr>
<th>Condition</th>
<th>-</th>
<th>-</th>
<th>+</th>
<th>+</th>
<th>+</th>
<th>-</th>
<th>-</th>
<th>-</th>
<th>-</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rapamycin</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>FKBP12.6-/-</td>
<td></td>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Gö6976</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Ryanodine</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

B

![Graph showing Phospho-eNOS Ser1177/eNOS Ratio](image)

- Con
- Rapa 1 umol/L
- FKBP12.6 -/-

**Phospho-eNOS Ser1177/eNOS Ratio (Arbitrary Units)**

+Go6976  +Ryanodine

* Statistical significance