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 FKBP12.6 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\textsuperscript{2+} release channels via rapamycin or genetic deletion causes a Ca\textsuperscript{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\textsuperscript{−/−} 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\textsuperscript{−/−} mice and in control aortas acutely treated in vitro with rapamycin to examine the direct vascular effects. In addition, we measured intracellular Ca\textsuperscript{2+} levels in rapamycin-treated and nontreated aortic endothelial cells from FKBP12.6\textsuperscript{−/−} and control mice. Rapamycin or genetic deletion of FKBP12.6 produced hypertension, as well as an endothelial intracellular Ca\textsuperscript{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\textsuperscript{−/−} (Pitzer, New London, CT), and FKBP12.6\textsuperscript{+/+} 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: TGGGCGGCGAACATGGGCTGAC; wild-type reverse: AAGCACTGCCCTCTGGAATAA; and wild-type reverse: CCCCCGGGTATGGATGTAGATAA. 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\textsubscript{2}PO\textsubscript{4}, 1.17 mmol/L of MgSO\textsubscript{4}·7H\textsubscript{2}O, 25 mmol/L of NaHCO\textsubscript{3}, 11.1 mmol/L of dextrose, and 2.5 mmol/L of CaCl\textsubscript{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\textsubscript{2}-5% CO\textsubscript{2}. All of the experiments were performed in the presence of indomethacin (10 \(\mu\)mol/L) to inhibit cyclooxygenase and to examine NO-mediated 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-response curves were obtained in a half-log, cumulative fashion to acetylcholine (ACH) and sodium nitroprusside after contraction to an EC\textsubscript{70} concentration of apocynin (10 \(\mu\)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.

**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 Ser1177 1:1000 (Upstate), 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. 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. Coomassie 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-Multimag 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. 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\textsuperscript{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\textsuperscript{2+} physiological salt solution (no CaCl\textsubscript{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\textsuperscript{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 (~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.

**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.
Figure 1. Rapamycin or FKBP12.6 gene deletion increased systolic arterial pressure. Control mice were treated with rapamycin (Rapa, 2 mg/kg per day) or vehicle for 7 days. Systolic blood pressures for FKBP12.6−/− mice were averaged from measurements taken on 2 separate days. Results are expressed as mean±SEM (n >3 per group). *P<0.05 vs control.

Statistical Analyses
Results are presented as mean±SEM. The Student’s t test was used to compare controls and controls plus either Gö6976 or ryanodine for measurements of NO production, eNOS protein expression, eNOS phosphorylation, PKC Ser660 phosphorylation, and intracellular Ca2+ levels. For measurements of blood pressure, vascular reactivity, NO production, eNOS protein expression, and eNOS phosphorylation, PKC Ser660 phosphorylation, and intracellular Ca2+ levels, an ANOVA was used for comparisons between groups followed by the Student–Newman–Keuls posthoc test when necessary. The significance level was 0.05.

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 Gö6976 (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). Gö6976 (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, Gö6976, 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 deletion 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
aortic NO production ≈70% compared with control aortas (l-NNa-sensitive peak DAF-FM fluorescent counts: FKBP12.6+/−=105 491±13 848; P<0.05 versus controls; Figure 4).

Because PKC is known to inhibit NO production, we verified that the improvement in relaxation responses induced by the cPKC-specific inhibitor G66976 was because of increased NO production. Isolated control aortas treated with rapamycin and nontreated aortas from FKBP12.6+/− mice were incubated with G66976 before homogenization. G66976 restored NO production in both groups (l-NNa-sensitive peak DAF-FM fluorescent counts: rapamycin + G66976 = 460 821 ± 49 878 and FKBP12.6+/− + G66976 = 414 578 ± 24 125; P>0.05 versus controls; Figure 4). G66976 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 FKBP12.6+/− mice to control levels (Figure 4).

**Effect of Rapamycin and FKBP12.6 Gene Deletion on eNOS and Phospho-eNOS Thr495 Expression**

eNOS protein levels were not different in rapamycin-treated control aortas or in aortas from FKBP12.6+/− mice compared with untreated controls (Figure 5). However, rapamycin or FKBP12.6 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 FKBP12.6+/− mice with the cPKC inhibitor G66976 or the RyR inhibitor ryanodine and measured eNOS Thr495 phosphorylation. Both G66976 and ryanodine reversed the increase in eNOS Thr495 phosphorylation by rapamycin or genetic deletion of FKBP12.6 (Figure 5). G66976 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 FKBP12.6 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 FKBP12.6+/− 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 vessels (P=0.089 for G66976). 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 FKBP12.6 Gene Deletion on PKC Activity**

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

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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 Ca2⁺ Levels
To examine whether a Ca2⁺ 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 Ca2⁺ release after pharmacological or genetic depletion of FKBP12/12.6. In primary MAECs from control mice, rapamycin caused a Ca2⁺ leak of 10% to 20% of the maximal ACh-induced Ca2⁺ release in nontreated control MAECs (Figure 7A). In response to ACh, rapamycin-treated MAECs had a markedly decreased peak Ca2⁺ release of 27±7% (P<0.05 versus controls) of controls followed by a sustained release of 20% to 25% (Figure 7B). This was in contrast to nontreated control MAECs, which demonstrated a much larger ACh-induced Ca2⁺ release, followed by a return to baseline. Similar to rapamycin-treated MAECs, MAECs from FKBP12.6/−/− mice demonstrated a peak ACh-induced Ca2⁺ release of 19±3% (P<0.05 compared with controls) followed by a sustained Ca2⁺ 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 Ca2⁺ leak. In MAECs from control mice, ryanodine abolished the intracellular Ca2⁺ leak after rapamycin (Figure 7A). Furthermore, ryanodine restored ACh-induced Ca2⁺ release in FKBP12.6/−/− MAECs to 80±10% of controls (Figure 7C). In the presence of ryanodine, rapamycin augmented ACh-induced Ca2⁺ 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 Ca2⁺ 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 Ca2⁺ release channels, because mice with a genetic deletion of FKBP12.6 demonstrate similarly altered intracellular Ca2⁺ 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 binding 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. 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). The phosphomimetic eNOS bound almost no calmodulin, and eNOS activity was abolished, except in the presence of supraphysiological concentrations of Ca^{2+} and calmodulin. 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

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, and interferes with the activation of eNOS by Ca\(^{2+}\)/calmodulin. In 1995, Hirata et al. reported that PKC directly phosphorylates eNOS, and down-regulation of PKC increased agonist-induced NO release 2-fold. Fleming et al. reported that PKC inhibition or down-regulation 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.

FKBP12/12.6 Depletion Induces an Intracellular Ca\(^{2+}\) Leak in Endothelial Cells

Because PKC can phosphorylate eNOS Thr495 leading to decreased calmodulin binding, eNOS activity, and NO production, alterations in intracellular Ca\(^{2+}\) and cPKC activation may have marked effects on endothelium-dependent dilation and blood pressure. PKC is activated at a lower concentration than eNOS, suggesting that the magnitude and localization of intracellular Ca\(^{2+}\) release play a major role in the activation of these enzymes. In the absence of extracellular Ca\(^{2+}\), we found that acute treatment of isolated endothelial cells with rapamycin produced an intracellular Ca\(^{2+}\) leak and decreased ACh-induced intracellular Ca\(^{2+}\) release. We were not able to observe a Ca\(^{2+}\) leak in endothelial cells from FKBP12.6/−/− mice; however, this leak was most likely compensated for by increased Ca\(^{2+}\) extrusion. This would result in a decrease in intracellular Ca\(^{2+}\) stores and most likely explains the decreased ACh-induced intracellular Ca\(^{2+}\) 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 Ca\(^{2+}\) 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 Ca\(^{2+}\) leaks from RyR may preferentially activate cPKC and alter eNOS phosphorylation. Whether RyRs and inositol triphosphate receptors control the same or distinct endoplasmic Ca\(^{2+}\) stores is controversial; however, our data suggest significant over-
lax, because inhibition of RyR opening in aortic endothelial cells from FKB12.6- knockout mice restored intracellular Ca\textsuperscript{2+} release induced by ACh, which releases Ca\textsuperscript{2+} via ryanodine-and inositol triphosphate-sensitive stores.\textsuperscript{26} Furthermore, Ca\textsuperscript{2+} release from RyRs appears to reduce intracellular Ca\textsuperscript{2+} stores, because ryanodine unmasked a rapamycin-induced augmentation in ACh-induced Ca\textsuperscript{2+} release. More studies are needed to clarify the contribution of RyRs to immunosuppressive drug-induced hypertension.

In conclusion, FKB12.6 contributes to endothelial function and blood pressure regulation by stabilizing endothelial intracellular calcium release channels. Rapamycin-induced displacement of FKB12.6 from RyRs or genetic deletion of FKB12.6 elevates blood pressure, which may be induced by ryanodine-sensitive intracellular Ca\textsuperscript{2+} store in fresh endothelial cells and evidence for ryanodine receptors. Circ Res. 1995;77:37–42.

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**Disclosures**

None.

**References**

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

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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 *in vitro* rapamycin (Rapa) treatment (1 μmol/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 ± SEM (n>3 mice for each group). *p<0.05 vs control.
Figure I

A

Phospho-eNOS-1177

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B

Phospho-eNOS Ser1177/eNOS Ratio
(Arbitrary Units)

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

* *