Removal of Fkbp12/12.6 From Endothelial Ryanodine Receptors Leads to an Intracellular Calcium Leak and Endothelial Dysfunction

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Objectives—FK506 Binding Protein 12 and its related isoform 12.6 (FKBP12/12.6) stabilize a closed state of intracellular Ca\(^{2+}\) release channels (ryanodine receptors [RyRs]), and in myocytes removal of FKBP12/12.6 from RyRs alters intracellular Ca\(^{2+}\) levels. The immunosuppressive drugs rapamycin and FK506 bind and displace FKBP12/12.6 from RyRs, and can also cause endothelial dysfunction and hypertension. We tested whether rapamycin and FK506 cause an intracellular Ca\(^{2+}\) leak in endothelial cells and whether this affects endothelial function and blood pressure regulation.

Methods and Results—Rapamycin or FK506 concentration-dependently caused a Ca\(^{2+}\) leak in isolated endothelial cells, decreased aortic NO production and endothelium-dependent dilation, and increased systolic blood pressure in control mice. Rapamycin or FK506 at 10 µmol/L abolished aortic NO production and endothelium-dependent dilation. Similar results were obtained in isolated endothelial cells and aortas from FKBP12.6\(^{-/-}\) mice after displacement of FKBP12 with 1 µmol/L rapamycin or FK506. In hypertensive FKBP12.6\(^{-/-}\) mice, systolic blood pressures were further elevated after treatment with either rapamycin or FK506. Blockade of the Ca\(^{2+}\) leak with ryanodine normalized NO production and endothelium-dependent dilation.

Conclusions—Complete removal of FKBP12 and 12.6 from endothelial RyRs induces an intracellular Ca\(^{2+}\) leak which may contribute to the pathogenesis of endothelial dysfunction and hypertension caused by rapamycin or FK506.

(Key Words: endothelium ■ hypertension ■ experimental ■ nitric oxide ■ ryanodine receptors)

The immunophilies FK506 Binding Proteins 12 and 12.6 (FKBP12/12.6) bind to intracellular Ca\(^{2+}\) release channels (ryanodine receptors [RyRs]) and stabilize a closed state of RyR. 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 FKBP12.6. In cardiac and skeletal myocytes, displacement of FKBP12/12.6 from RyRs or mutations that alter FKBP12/12.6-RyR interactions create a Ca\(^{2+}\) leak by increasing the probability and duration of RyR opening. This leak may lead to fatal cardiac arrhythmias or skeletal muscle diseases such as malignant hyperthermia and central core disease.

The immunosuppressive drugs rapamycin or FK506 bind FKBP12/12.6 and the rapamycin-FKBP12/12.6 complex inhibits mammalian target of rapamycin (mTOR) whereas the FK506-FKBP12/12.6 complex inhibits calcineurin. Although rapamycin and FK506 markedly increase the success of organ transplantation, they both can cause endothelial dysfunction and hypertension. Furthermore, treatment of rats with FK506 decreases production of the potent endothelium-derived vasodilator nitric oxide (NO), and genetic deletion of FKBP12.6 in mice causes hypertension. These findings suggest the possibility that depletion of vascular FKBP12/12.6, and thus an endothelial intracellular Ca\(^{2+}\) leak, may alter endothelial function and blood pressure regulation, however this has not been studied previously.

We hypothesized that genetic and/or pharmacological displacement of endothelial FKBP12 and 12.6 from RyRs causes a Ca\(^{2+}\) leak which leads to decreased NO production, decreased endothelium-dependent dilation, and increased blood pressure. To test this we measured intracellular Ca\(^{2+}\) levels in rapamycin- or FK506-treated primary aortic endothelial cells from control and FKBP12.6\(^{-/-}\) mice. We also measured peak NO production, NO release, and endothelium-dependent dilation in control and FKBP12.6\(^{-/-}\) aortas after acute in vitro treatment with rapamycin or FK506 to examine the direct vascular effects. Additionally, we measured systolic blood pressures in control and FKBP12.6\(^{-/-}\) mice treated for 7 days with rapamycin or FK506.

Methods

Animals and Blood Pressure Measurements
Male C57Bl/6 (Harlan; Indianapolis, Ind), FKBP12.6\(^{-/-}\) (Pfizer; New London, Conn), and FKBP12.6\(^{-/-}\) mice aged 10 to 18 weeks...
were used in all experiments. FKBP12.6 mice were genotyped using tail DNA and the following primers (5′ to 3′): mutant Forward-1108: TGGCCGGCGAATGCGTGCAG; mutant Reverse-TGGGGCAC-1109: ACTGGCGGACAC; wild-type Forward-AAGCAGTCCGGTCT-1110: GGAATATAA; wild-type Reverse-CGCCGGGATGAAAGTGTTAGAAT. All procedures were approved by the Baylor College of Medicine’s Institutional Animal Care and Use Committee. Tail-cuff systolic blood pressures (ITC, Inc) were measured at baseline and after 7 days of treatment with rapamycin (0.1, 2, or 10 mg/kg/d, i.p.), FK506 (0.1, 2, or 10 mg/kg/d, i.p.), or DMSO vehicle (0.2% final concentration). We determined previously that there were no differences in blood pressure, endothelium-dependent dilatation, or aortic NO production between C57Bl/6 and FKBP12.6+/− mice (background = C57Bl/6) therefore aortas from these mice were grouped together as controls.

**Intracellular Ca2+ Imaging**

Primary mouse aortic endothelial cells (MAECs) were isolated using Matrigel, and Ca2+ imaging was performed as described previously.17,18 MAECs were loaded with Fura-2 AM (10 μmol/L) diluted in a physiological salt solution (PSS; 119.0 mmol/L NaCl, 4.7 mmol/L KCl, 1.18 mmol/L KH2PO4, 1.17 mmol/L MgSO4·H2O, 25 mmol/L NaHCO3, 11.1 mmol/L dextrose, and 2.5 mmol/L CaCl2) and were imaged on a Nikon Eclipse E600FN equipped with a Cascade 512B charge-coupled device (CCD) camera (Photometrics). Cells were placed in zero-calcium PSS (no CaCl2, 100 μmol/L EGTA) or PSS, and the ratio of 340/380 nm was monitored continuously for >10 endothelial cells. In parallel experiments, endothelial cells were pretreated with the RyR inhibitor ryanodine (50 μmol/L, 60 minutes). After 5 minutes, cells were treated with either rapamycin or FK506 (0.1 to 10 μmol/L) for 15 minutes. To increase Ca2+ mobilization from intracellular stores, cells were treated with ace-tylcholine (ACh, 1 μmol/L) and monitored for 5 minutes.

**Preparation of Vascular Homogenates**

Endothelium-intact aortic rings were incubated in the absence and presence of rapamycin or FK506 (0.1 to 10 μmol/L, 20 minutes) and/or ryanodine (50 μmol/L, 60 minutes) before homogenization. Vascular homogenates were centrifuged at 11 000 rpm for 10 minutes at 4°C and protein concentrations were determined by Lowry assay using bovine serum albumin as the standard.19

**Peak NO Production**

An assay using the cell-permeable dye 4-aminomethyl-2′,7′-difluorofluorescein diacetate (DAF-FM diacetate; Invitrogen Molecular Probes) was used to measure peak NO production as described previously.18 In brief, 20 μg of protein, 12 μmol/L 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 N′-nitro-L-arginine (L-NNA; 100 μmol/L, 20 minutes). Peak 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.

**Aortic NO Release**

DAF-FM diacetate (Invitrogen Molecular Probes) was also used to measure NO release from aortic rings. The fluorescence of PSS and 6 μmol/L DAF-FM diacetate, stirred continuously and warmed to 37°C, was recorded for 10 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 3 per minute. One aortic ring (3 mm) was added to the cuvette and fluorescent counts were measured for 5 minutes. ACh (10 μmol) was then added and fluorescent counts were measured for 5 minutes. Basal and agonist-induced NO release was determined by measuring peak fluorescent counts minus the peak fluorescent counts of PSS and DAF-FM diacetate (no aortic ring) and is expressed as percent of controls.

**Vascular Reactivity**

Vascular reactivity was measured as described previously.18 All experiments were performed in the presence of indomethacin (10 μmol/L) to inhibit cyclooxygenase. To examine the direct effects on endothelium-dependent relaxation responses, aortic rings were incubated with rapamycin or FK506 (0.1 to 10 μmol/L) for 20 minutes. Concentration-force curves were obtained in a half-log, cumulative fashion to ACh and sodium nitroprusside (SNP) after contraction to an EC50 concentration of phenylephrine (PE; 1 μmol/L). Relaxation responses were also assessed following the NOS inhibitor N′-nitro-L-arginine (L-NNA; 10 μmol/L, 20 minutes) or the RyR inhibitor ryanodine (50 μmol/L, 60 minutes).

**Statistical Analyses**

Results are presented as mean±SEM. The Student t test was used to compare variables between 2 groups. An analysis of variance was used for multiple comparisons followed by the Student-Newman-Keuls post hoc test when necessary. The significance level was 0.05.

**Results**

**Effects of FKBP12.6 Gene Deletion and/or Rapamycin/FK506 on Intracellular Ca2+ Levels**

To examine whether a Ca2+ leak induced by the displacement of FKBP12/12.6 from RyRs occurs in endothelial cells we measured endothelial intracellular Ca2+ release after genetic and/or pharmacological depletion of FKBP12/12.6 in the absence of extracellular Ca2+. In primary MAECs from control mice, FK506 caused a concentration-dependent intracellular Ca2+ leak (at 5 minutes: FK506 0.1 μmol/L = 8±2%, FK506 1 μmol/L = 20±3%, and FK506 10 μmol/L = 27±3% of the maximal ACh-induced Ca2+ release in non-treated control MAECs; Figure 1A). Similar concentration-dependent results were obtained with rapamycin (some data not shown) as 1 μmol/L caused an intracellular Ca2+ leak of 15±3% (Figure 1B). In primary MAECs from FKBP12.6−/− mice, administration of 1 μmol/L rapamycin to displace the remaining FKBP12 from RyRs caused an intracellular Ca2+ leak of 26±3% of the maximal ACh-induced Ca2+ release in nontreated control MAECs (Figure 1B).

Because FKBP12/12.6 binds to RyR and stabilizes a closed state of the channel, we tested whether inhibition of RyR opening with ryanodine could block the Ca2+ leak. In MAECs from control mice ryanodine abolished the intracellular Ca2+ leak after FK506 (data not shown). Similarly, ryanodine blocked the intracellular Ca2+ leak induced by rapamycin in endothelial cells from control and FKBP12.6−/− mice (Figure 1B).

It is possible that the rapamycin/FK506-induced intracellular Ca2+ leak may elicit a Ca2+ influx, therefore we measured intracellular Ca2+ levels after rapamycin or FK506 in the presence of extracellular Ca2+. Figure 2 shows that FK506 caused a concentration-dependent intracellular Ca2+ leak as shown in Figure 1A, however we did not observe a further increase in intracellular Ca2+ levels (in the form of a sharp rise attributable to the concentration gradient) in response to this leak. The same result occurred in MAECs from FKBP12.6−/− mice (Figure 2). These findings suggest that a Ca2+ influx did not occur as a result of the intracellular Ca2+ leak.
Effects of FKBP12.6 Gene Deletion and/or Rapamycin/FK506 on NO Production

Acute in vitro treatment of control aortas with rapamycin decreased peak NO production in a concentration-dependent manner (Figure 3A). Rapamycin at 0.1 μmol/L decreased peak aortic NO production 40%, 1 μmol/L rapamycin decreased peak aortic NO production 79%, and 10 μmol/L rapamycin decreased peak aortic NO production 94% compared with untreated controls (L-NNA-sensitive peak DAF-FM fluorescent counts: FK506 0.1 μmol/L = 206285 ± 12540, rapamycin 1 μmol/L = 37338 ± 64780, FK506 10 μmol/L = 19961 ± 9941, and controls = 345764 ± 56478, P<0.05 versus controls; Figure 3A). Almost identical results were obtained with FK506 (data not shown). Complete removal of FKBP12 and 12.6 from RyRs by genetic deletion of FKBP12.6 plus 1 μmol/L rapamycin or FK506 abolished peak aortic NO production, similar to control vessels treated with 10 μmol/L rapamycin or FK506 (L-NNA-sensitive peak DAF-FM fluorescent counts: FKBP12.6−/− = 105491 ± 13848, FKBP12.6−/− + rapamycin = 26296 ± 4502, FKBP12.6−/− + FK506 = 20054 ± 3532, and controls = 345764 ± 56478, P<0.05 versus controls; Figure 3B).

We also measured basal and agonist-induced NO release in aortic rings from control and FKBP12.6−/− mice. Rapamycin or FK506 concentration-dependently decreased basal NO release (Figure 4A). Aortas from FKBP12.6−/− mice also exhibited significantly decreased basal NO release compared with controls (Figure 4B), and basal NO release was abolished after 1 μmol/L rapamycin or FK506. Similar results for ACh-induced NO release were found in aortas from control and FKBP12.6−/− mice after rapamycin or FK506 (Figure 4C and 4D).

Effects of FKBP12.6 Gene Deletion and/or Rapamycin/FK506 on Endothelium-Dependent and -Independent Relaxation

Rapamycin or FK506 decreased maximal aortic relaxation responses to ACh in a concentration-dependent manner (Figure 5A). Ten μmol/L rapamycin or FK506 abolished relaxation responses to ACh (relaxation from PE-induced contraction: rapamycin 10 μmol/L = 13 ± 3%, FK506 10 μmol/L = 7 ± 3%, and controls = 83 ± 3%; P<0.05 versus controls, Figure 5A). Maximal relaxation responses were also abolished in aortas from FKBP12.6−/− mice after removal of FKBP12 with 1 μmol/L rapamycin- or FK506 (relaxation...
rapamycin 2 mg/kg/d: from 113±5 mm Hg to 138±3 mm Hg, rapamycin 10 mg/kg/d: from 96±1 mm Hg to 152±1 mm Hg, and vehicle-treated controls: from 107±3 mm Hg to 108±3 mm Hg; all P<0.05 versus controls, Figure 6). Similar results were found with FK506 (data not shown). Systolic blood pressure was 142±2 mm Hg in FKBP12.6−/− mice (Figure 6), which was significantly higher than controls and similar to the 145±2 mm Hg reported previously.16 When FKBP12.6−/− mice were treated with 2 mg/kg/d rapamycin or FK506 for 7 days, systolic blood pressure increased further to 151±1 mm Hg and 157±2 mm Hg, respectively.

Discussion
Alterations in intracellular Ca2+ homeostasis affect cellular function; however the functional effects of an endothelial intracellular Ca2+ leak are unknown. The present study demonstrates that genetic and/or pharmacological displacement of FKBP12/12.6 from endothelial RyRs leads to an intracellular Ca2+ leak which in turn decreases NO production and endothelium-dependent dilation. Furthermore, depletion of endothelial FKBP12 and 12.6 may be partly responsible for the increase in systolic arterial pressure.

The role of RyRs in endothelial cell Ca2+ homeostasis is not well defined. Rapamycin or FK506 binding of FKBP12/12.6 and displacement from RyRs leads to a concentration-dependent intracellular Ca2+ leak, similar to that which occurs in myocytes.3–5,8,9 Rapamycin or FK506 also concentration-dependently decreased peak aortic NO production, basal and agonist-induced aortic NO release, and endothelium-dependent dilation. This supports the findings of Takeda and colleagues who reported decreased eNOS activity in aortic endothelial cells from rats treated with FK506 (5 mg/kg/d) for 4 weeks.15 In the current study, acute in vitro treatment of aortas with a high concentration of rapamycin or FK506 (10 μmol/L) abolished aortic NO production and endothelium-dependent dilation demonstrating a direct vascular effect. These data suggest that complete removal of FKBP12/12.6 from endothelial RyRs markedly alters intracellular Ca2+ release and decreases endothelial function. Furthermore, the degree of intracellular Ca2+ leak was negatively correlated with peak aortic NO production (-0.988, P<0.05), basal aortic NO release (-0.985, P<0.05), and endothelium-dependent dilation (-0.998, P<0.05).

Supportive evidence comes from our findings in mice lacking FKBP12.6. Removal of the remaining FKBP12 with 1 μmol/L rapamycin or FK506 caused an intracellular Ca2+ leak that resembled that of control aortic endothelial cells treated with 10 μmol/L rapamycin or FK506. Additionally, genetic deletion of FKBP12.6 plus 1 μmol/L rapamycin or FK506 abolished peak aortic NO production, basal and agonist-induced aortic NO release, and endothelium-dependent dilation. Inhibition of RyR opening prevented the endothelial cell Ca2+ leak and normalized peak NO production and endothelium-dependent dilation following FKBP12/12.6 depletion. Taken together, these findings suggest that an endothelial RyR-mediate Ca2+ leak detrimentally affects endothelial function by decreasing NO production.

Effects of FKBP12.6 Gene Deletion and/or Rapamycin/FK506 on Systolic Blood Pressure
Rapamycin treatment for 7 days concentration-dependently increased systolic arterial pressure in control mice (rapamycin 0.1 mg/kg/d: from 103±5 mm Hg to 121±2 mm Hg, from PE-induced contraction: FKBP12.6−/− + 1 μmol/L rapamycin =1.13±7%, FKBP12.6−/− + 1 μmol/L FK506 = 10±5%; P<0.05 versus controls, Figure 5B). NOS inhibition with L-NNa (10 μmol/L) abolished relaxation responses to ACh in all conditions (data not shown). Inhibition of RyR opening with ryanodine (50 μmol/L) restored relaxation responses in rapamycin- or FK506-treated control aortas (data not shown) and in rapamycin- or FK506-treated aortas from FKBP12.6−/− mice (Figure 5B), but had no significant effect on relaxation responses in untreated control aortas. The improvements in ACh-induced relaxation after ryanodine were blocked by L-NNa (data not shown). Endothelium-independent relaxation responses to SNP were not different in rapamycin- or FK506-treated control aortas or in treated aortas from FKBP12.6−/− mice compared with controls (data not shown).
Endothelium-derived nitric oxide (NO) plays a major role in vascular tone and blood pressure regulation. The formation of NO from L-arginine via the enzymatic activity of endothelial NO synthase (eNOS) is dependent on Ca$^{2+}$/calmodulin and is regulated in part by its phosphorylation status. The attenuation of endothelial NO biosynthesis and endothelium-dependent dilation by genetic and/or pharmacological depletion of FKBP12/12.6 may be attributable to several factors. One factor may be a change in eNOS protein expression. However, because we performed acute in vitro incubations with rapamycin and FK506 we would not expect protein levels to change in this short amount of time, and aortic eNOS protein levels were not reduced in FKBP12.6$^{-/-}$ mice compared with controls. Second, the Ca$^{2+}$ leak may preferentially activate kinases and/or phosphatases that affect eNOS phosphorylation. Protein kinase C (PKC) is the most likely kinase for coupling minor changes in cytoplasmic Ca$^{2+}$ to decreased eNOS activity as PKC is activated by a lower concentration of intracellular Ca$^{2+}$ ($EC_{50}=\approx 200$ μmol/L) than eNOS ($EC_{50}=\approx 400$ μmol/L) and is known to phosphorylate eNOS at an inhibitory site, threonine 495. We previously reported that rapamycin or FKBP12.6 gene deletion increases PKC-mediated eNOS threonine 495 phosphorylation and decreases endothelial function, and these effects were reversed by ryanodine or a PKC inhibitor. Another possibility is that the intracellular Ca$^{2+}$ leak elicited by removal of FKBP12/12.6 from endothelial RyRs may alter the subplasmalemmal Ca$^{2+}$ levels necessary to activate caveolae-bound eNOS. Whether or not pharmacological and/or genetic depletion of FKBP12/12.6 alters plasma membrane Ca$^{2+}$ channel function and agonist-induced Ca$^{2+}$ influx is currently being investigated. Taken together, our data suggest that an endothelial RyR-mediated Ca$^{2+}$ leak alters intracellular Ca$^{2+}$ levels and eNOS phosphorylation, both of which may contribute to the decreased NO biosynthesis and endothelium-dependent dilation following depletion of endothelial FKBP12/12.6.

Rapamycin and FK506 are known to cause endothelial dysfunction and hypertension in organ transplant recipients, however the mechanisms remain unknown. Also, it was unknown whether rapamycin and FK506 increase blood pressure by direct or indirect vascular effects and whether the displacement of FKBP12/12.6 from RyRs plays a role. Xin and colleagues first showed that genetic deletion of FKBP12.6 causes hypertension in mice supporting a role for FKBP12/12.6. We found that rapamycin or FK506 treatment of control mice increased systolic blood pressure in a concentration-dependent manner, and this...
correlated negatively with aortic NO production and endothelium-dependent dilation after acute in vitro treatment with rapamycin or FK506. Takeda and colleagues also reported that 5 mg/kg/d treatment of rats for 2 or 4 weeks elevated systolic blood pressures significantly, but 0.5 mg/kg/d had no effect.\textsuperscript{13} Treatment of hypertensive FKBP12.6\textsuperscript{-/-} mice with 2 mg/kg/d rapamycin or FK506 further increased systolic blood pressure; these values were similar to that of control mice treated with 10 mg/kg/d rapamycin or FK506. Our findings in FKBP12.6\textsuperscript{-/-} mice suggest that FKBP12 plays a compensatory role, and the augmentation of blood pressure by rapamycin or FK506 may be attributable to the displacement of FKBP12 from RyRs and abolishment of endothelial NO production and endothelium-dependent dilation. Thus, alterations in endothelial intracellular Ca\textsuperscript{2+} levels have marked effects on endothelium-dependent dilation and blood pressure and may explain why hypertension is evident in mice with a genetic deletion of FKBP12.6, rats treated with FK506, and humans treated with rapamycin or FK506. We cannot rule out that FKBP12/12.6 depletion also affects vascular smooth muscle function which may contribute to the hypertension. We have found that rapamycin or FK506 did not contract denuded aortas from control mice (data not shown), however contractions to phenylephrine were slightly potentiated by rapamycin or FK506. This supports previous studies which reported that FK506 did not contract various bovine arteries, but increased the sensitivity to norepinephrine in human and rat resistance arteries.\textsuperscript{24,25} Previous studies have also shown that FKBP12/12.6 modulates Ca\textsuperscript{2+} release in smooth muscle cells.\textsuperscript{26-28} Tang et al demonstrated that FK506 dose-dependently increased the open probability of bovine coronary artery smooth muscle cell RyRs reconstituted in lipid bilayers.\textsuperscript{26} Ji et al found increased frequency and amplitude of spontaneous Ca\textsuperscript{2+} sparks in urinary bladder smooth muscle cells from FKBP12.6\textsuperscript{-/-} mice.\textsuperscript{27} Thus it is possible that increased smooth muscle contraction in addition to decreased endothelial NO production may explain the hypertension in our rapamycin/FK506-treated control and FKBP12.6\textsuperscript{-/-} mice. In conclusion, FKBP12/12.6 contributes to endothelial function and blood pressure regulation by stabilizing endothelial intracellular Ca\textsuperscript{2+} release channels. Displacement of FKBP12/12.6 from endothelial RyRs induces an intracellular Ca\textsuperscript{2+} leak and hypertension which may be mediated by decreased endothelial NO production and endothelium-dependent dilation. Prevention of endothelial intracellular Ca\textsuperscript{2+} leaks may represent a novel therapeutic target to prevent the endothelial dysfunction and hypertension associated with rapamycin and FK506.

**Figure 6.** Systolic arterial pressure increased in control mice treated with rapamycin (Rapa, 0.1, 2, or 10 mg/kg/d) for 7 days. Systolic arterial pressure also increased in FKBP12.6\textsuperscript{-/-} mice treated with either rapamycin (Rapa, 2 mg/kg/d) or FK506 (2 mg/kg/d) for 7 days. \textsuperscript{*}P<0.05 vs control, \textsuperscript{†}P<0.05 vs Rapa 0.1 mg/kg, \textsuperscript{‡}P<0.05 vs 12.6\textsuperscript{-/-}.

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**Disclosures**

None.
References

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