3-Hydroxymethyl coenzyme A reductase inhibition attenuates spontaneous smooth muscle tone via RhoA/ROCK pathway regulated by RhoA prenylation

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Rattan S. 3-Hydroxymethyl coenzyme A reductase inhibition attenuates spontaneous smooth muscle tone via RhoA/ROCK pathway regulated by RhoA prenylation. Am J Physiol Gastrointest Liver Physiol 298: G962–G969, 2010. First published April 8, 2010; doi:10.1152/ajpgi.00034.2010.—RhoA prenylation may play an important step in the translocation of RhoA in the basal internal anal sphincter (IAS) smooth muscle tone. Statins inhibit downstream posttranslational RhoA prenylation by 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibition (HMGCRI). The role of statins in relation to RhoA prenylation in the pathophysiology of the spontaneously tonic smooth muscle has not been investigated. In the present studies, we determined the effect of classical HMGCRI simvastatin on the basal IAS tone and RhoA prenylation and in the levels of RhoA/Rho kinase (ROCK) in the cytosolic vs. membrane fractions of the smooth muscle. Simvastatin produced concentration-dependent decrease in the IAS tone (via direct actions at the smooth muscle cells). The decrease in the IAS tone by simvastatin was associated with the decrease in the prenylation of RhoA, as well as RhoA/ROCK in the membrane fractions of the IAS, in the basal state. The inhibitory effects of the HMGCRI were completely reversible by geranylgeranyltransferase substrate geranylgeranyl pyrophosphate. Relaxation of the IAS smooth muscle via HMGCRI simvastatin is mediated via the downstream decrease in the levels of RhoA prenylation and ROCK activity. Studies support the concept that RhoA prenylation leading to RhoA/ROCK translocation followed by activation is important for the basal tone in the IAS. Data suggest that the role of HMG-CoA reductase may go beyond cholesterol biosynthesis, such as the regulation of the smooth muscle tone. The studies have important implications in the pathophysiological mechanisms and in the novel therapeutic approaches for anorectal motility disorders.

internal sphincter; mevalonate; geranylgeranyltransferase; Rho kinase

SPONTANEOUS TONE in the internal anal sphincter (IAS) smooth muscle is considered to be primarily myogenic in nature and plays a crucial role in anorectal continence (20, 27). Hypertensive IAS has been associated with motility disorders like Hirschsprung’s disease (22, 27), recurrent anal fissures, and hemorrhoids (7, 19). Hypotensive IAS on the other hand results in rectoanal incontinence (20). Molecular mechanisms for the regulation of IAS tone are not completely understood. Such information is important for the understanding of the pathophysiology of the above abnormalities and their therapeutic management.

Activation of Ser/Thr kinase Rho kinase (ROCK) by GTP-RhoA is a critical step in RhoA-ROCK-mediated Ca$^{2+}$ sensitization in the smooth muscle. RhoA cycles between a biologically inactive GDP-bound state and an active GTP-bound state. Thus, in resting state, Rho GDP dissociation inhibitor (Rho GDI) binds to GDP-RhoA and extracts GDP-RhoA from the membrane to the cytosol (36). With the agonist stimulation of G protein-coupled receptors (GPCRs), guanine nucleotide exchange factors convert GDP-RhoA to GTP-RhoA. GTP-RhoA associates with the plasma membrane via its prenylated tail, leading to the activation of ROCK (14).

It follows, therefore, that RhoA prenylation may be an important step for the translocation of RhoA to the cellular membrane. RhoA is posttranslationally modified by the isoprenoid lipid geranylgeranyl (6). In addition, prenyltransferase, geranylgeranyltransferase I (GGTase I), catalyzes the covalent attachment of the geranylgeranyl group from geranylgeranyl pyrophosphate (GGPP) to the carboxyl-terminal cysteine of RhoA (39).

Commonly used lipid-lowering agents or statins work via inhibition of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, the rate-limiting enzyme in the biosynthesis of cholesterol in the liver. HMG-CoA reductase catalyzes the conversion of HMG-CoA to mevalonic acid. In addition to inhibiting cholesterol synthesis, statins also block the synthesis of isoprenoid intermediates such as farnesyl pyrophosphate and GGPP (13). By inhibiting these isoprenoid intermediates, statins prevent the prenylation of GTPases including RhoA. Because of this prominent effect, actions of statins may go beyond the inhibition of cholesterol biosynthesis. Consequently, HMG-CoA reductase may play an important role not only in the cholesterol biosynthesis but also in the increased smooth muscle contractility (6), neurogenic relaxation of the smooth muscle via G protein coupling (33), neuroinflammation, neurological diseases and stroke (40), and embryonic development (23).

The purpose of the present investigation was to determine the upstream regulation of RhoA prenylation via HMG-CoA reductase in the IAS tone using classical HMG-CoA reductase inhibition (HMGCRI) simvastatin. In this regard, we determined the levels and cellular distribution of prenylated vs. nonprenylated RhoA ROCK II and ROCK activity in the basal state of the IAS, before and after HMGCRI simvastatin. For direct comparison of this regulation, some studies were performed in the adjoining nontonic smooth muscle of the rectum (RSM).

MATERIALS AND METHODS

Tissue preparation. The studies were performed in the spontaneously tonic smooth muscle of the IAS and the adjoining nontonic smooth muscle, the RSM. Sprague-Dawley rats (300–350 g) were euthanized by decapitation. The anorectal tissues were then quickly removed and transferred to oxygenated (95% O$_2$-5% CO$_2$) Krebs physiological solution (KPS) of the following composition (in mmol):
118.07 NaCl, 4.69 KCl, 2.52 CaCl₂, 1.16 MgSO₄, 1.01 NaH₂PO₄, 25 NaHCO₃, and 11.10 glucose (37°C). Circular smooth muscle strips (∼0.5 × 7 mm) of the IAS and the RSM were prepared as explained previously (29).

Pretreatment of the smooth muscle tissues with simvastatin. The IAS and RSM strips were cultured in Leibovitz medium (L-15) (25/29/0.5) previously (29). Decrease in the basal IAS tone was expressed as a percentage of maximal decrease caused by 10 mM EDTA, and increase in the tone was expressed as a percentage of maximal by 300 μM bethanecol, determined at the end of each experiment (5).

The experimental protocol of the study was approved by the institutional Animal Care and Use Committee of Thomas Jefferson University and was in accordance with the recommendations of the American Association for the Accreditation of Laboratory Animal Care.

Drug responses. Changes in the basal IAS tone and the cumulative concentration-response curves (CRCs) for U-46619 (1 nM to 10 μM) in the IAS strips were determined before and after pretreatment with simvastatin (0.1 to 10.0 μM). To determine the reversibility by GGTase substrate, some experiments were performed with the simultaneous incubations of the smooth muscles with GGPP (10 μM) plus simvastatin.

Immunoprecipitation of prenylated proteins and Western blot analysis. Following the incubation with either simvastatin or the vehicle, the smooth muscle tissue strips were frozen in liquid N₂ and immediately stored at −80°C. At appropriate time, the frozen tissues were cut into small pieces, and homogenization buffer (50 mM HEPES, pH 7.5, 140 mM NaCl, 1 mM EDTA, 1% Triton X-100, 2 mM NaVO₄, 25 mM NaF) was added in a volume equal to five times the weight of the tissues. The mixture was then homogenized on ice. The homogenates were then centrifuged (14,000 revolution/min) for 5 min, and supernatants were collected. Protein concentration in resultant supernatants was determined by the method of Lowry et al. (18) using BSA as a standard (Pierce Biotechnology, Rockford, IL). Preyalted RhoA was immunoprecipitated using Roche Diagnostics immuno precipitation kit (Protein G) (Fisher, Allentown, PA), following manufacturer’s instructions. Briefly, 200 μg of tissue lysate in 250 μl volume was precleared with 25 μl protein G agarose beads. Precleared lysate was incubated with 1 μg of anti-farnesyl rabbit polyclonal antibody (Calbiochem, San Diego, CA) for 1 h (4). Then 25 μl of protein G agarose beads were added and further incubated overnight to immobilize prenylated proteins. Agarose beads were centrifuged at 20 s at 10,000 g, and supernatants were transferred to a fresh tube for a fraction containing unprenylated proteins. Agarose beads were washed repeatedly with wash buffer (50 mM Tris·HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P40, 0.5% sodium deoxycholate). Later, 50 μl Lamellose sample buffer (LSB; with final concentration of 62.5 mM Tris, 1% SDS, 15% glycerol and 0.005% bromphenol blue, and 2% β-mercaptoethanol) was added to the beads and placed in a boiling water bath for 5 min. Twenty micrograms of unprenylated fraction was also similarly added with LSB. Protein samples were separated by 15% SDS-polyacrylamide gel.

The proteins separated above were electrophoretically transferred onto a nitrocellulose membrane at 100 V for 1 h at 4°C. To block nonspecific antibody binding, the membranes were soaked overnight at 4°C in Tris-buffered saline with Tween (TBS-T; composed of: 20 mM Tris pH 7.6, 137 mM NaCl, and 0.1% Tween-20) containing 5% nonfat dry milk. The membrane was then incubated with the RhoA primary antibody raised in rabbit (1:1,000 diluted in TBS-T containing 1% milk) for 1 h at room temperature. After being washed with TBS-T three times (10 min each wash), the membranes were incubated with the horseradish peroxidase-conjugated bovine anti-rabbit secondary antibody (1:10,000). The membranes were washed three times with TBS-T, and the corresponding bands were visualized with enhanced chemiluminescence substrate using the SuperSignal West Pico Chemiluminescent Substrate (Pierce Biotechnology) and Hyperfilm MP (Amersham Bioscience, Piscataway, NJ). Bands corresponding to different proteins on X-ray films were scanned with a scanner (model SNAPSHOT 310; Agfa, Ridgefield Park, NJ), and their relative densities were determined by using Image-Pro Plus 4.0 software (Media Cybernetics, Silver Spring, MD).

Cytosolic and particulate fractions. The IAS and RSM tissues incubated with the vehicle (control) as well as HMG-CoA reductase inhibitor were used for cytosolic and particulate fraction collections. For this, the respective tissues were homogenized in ice-cold homogenization buffer (10 mM Tris·HCl, pH 7.5, 5 mM MgCl₂, 2 mM EDTA, 250 mM sucrose, and 1 mM dithiothreitol). The homogenates were centrifuged at 10,000 g for 30 min at 4°C (L-8–70M Ultracentrifuge; Beckman, Fullerton, CA). The supernatants were then transferred to a fresh tube and used as the cytosolic fraction. Pellets containing membrane proteins were resuspended in homogenization buffer containing 1% Triton X-100 and homogenized. The pellet extract was centrifuged at 800 g for 10 min, and the supernatant was collected as the particulate fraction (12).

Above prepared protein extracts (20 μg) were mixed with LSB and separated by 15% SDS-polyacrylamide gel. The separated proteins were electrophoretically transferred onto a nitrocellulose membrane, RhoA Western blots were performed as described before (30), and bands were captured on X-ray film. Nitrocellulose membranes were stripped of secondary and primary antibodies by incubating with Restore Western blot stripping buffer (Pierce Biotechnology) for 15 min at room temperature and then reprobed for α-actin as described earlier (10).

ROCK activity assay. ROCK activity was determined by monitoring the levels of phosphorylation of the endogenous ROCK substrate regulatory subunit of myosin light-chain phosphatase (MYPT1) in total IAS smooth muscle tissue extracts using a rat anti-phospho-Thr696 antibody (17). For this, the IAS tissues were pretreated with different concentrations (0.1 to 10.0 μM) of simvastatin and (as explained above) and 10 μM of selective ROCK inhibitor Y 27632.

Preparation of dispersed IAS smooth muscle cells and measurement of cell lengths. Smooth muscle cells (SMCs) were isolated from the IAS smooth muscle by sequential enzymatic digestion, filtration, and centrifugation as described previously (10). Briefly, the IAS smooth muscle strips were incubated in KPS containing 0.1% collagenase and 0.01% trypsin inhibitor. The partly digested strips were washed, and SMCs were allowed to disperse spontaneously for 30 min at room temperature and then collected in 1% Tryton X-100 and centrifuged at 350 g for 10 min. The cells were cultured in 10-cm plates in DMEM containing 10% fetal bovine serum, 5% penicillin-streptomycin, 50 μg/ml gentamycin, and 2 μg/ml amphotericin B at 37°C with 5% CO₂. The cells were then incubated with simvastatin (0.1, 1.0, and 10.0 μM) for 24 h. Control cells were incubated with the vehicle solution only. Individual cell length was measured by computerized image microscopy. The average length of cells in the control state or with a test agent was obtained as described earlier (10).
Comparing more than two groups. CRC curves were analyzed using two-way ANOVA test.

**Results**

**Effect of HMGCoA reductase inhibitor on the basal IAS tone.** Pretreatment of the IAS smooth muscle strips with HMGCoA reductase inhibitor caused concentration-dependent decrease in the basal tone. Simvastatin (10 μM) caused 62.5 ± 6.4% inhibition in the basal IAS tone (*P < 0.05; n = 6; Fig. 1). These results were compared with the non-simvastatin-treated smooth muscle strips (incubated with the vehicle only, considered as controls).

The inhibitory effect of simvastatin was reversed following GGTase substrate GGPP (10 μM) so that the values obtained following the combined treatment were not significantly different from those of controls (*P > 0.05; n = 6; Fig. 1).

**Effect of HMGCoA reductase inhibitor on the cellular distribution of RhoA in the IAS vs. the RSM SMCs.** To demonstrate the site of action of the HMGCoA reductase inhibitor on the isolated SMC from the IAS vs. RSM. Data show that simvastatin caused concentration-dependent greater increase in the lengths of the SMC from the IAS compared with those from the RSM (*P < 0.05; Fig. 2). Simvastatin (10 μM) caused maximal relaxation of 28.9 ± 3.8% of the IAS SMCs compared with 6.2 ± 2.3% in the case of the RSM.

**Effect of simvastatin on U-46619-induced increase in the IAS tone.** Thromboxane A2 analog U-46619 caused concentration-dependent increase in IAS tone (the maximal effect, E_max = 59.9 ± 5.1%, with 3 × 10^-6 M; EC_50 = 2.5 × 10^-8 M; n = 6). Pretreatment with simvastatin caused significant attenuation of these responses (E_max = 25.5 ± 5.5%; EC_50 = 3.2 × 10^-6 M; P < 0.05; n = 4, Fig. 3A).

The suppressant effect of simvastatin on U-46619-induced increase in the IAS tone was reversed by 10 μM GGPP so that U-46619 CRC examined in the simvastatin + GGPP (both 10 μM) group was not significantly different from control (*P > 0.05; Fig. 3B).

**Effect of HMGCoA reductase inhibitor on RhoA prenylation.** Prenylated proteins were immunoprecipitated using anti-farnesyl rabbit antibody (4). The precipitates containing prenylated proteins were then specifically analyzed for the levels of prenylated RhoA via Western blot using RhoA antibody. Similarly, supernatants (containing unprenylated proteins) were analyzed for unprenylated RhoA. Whereas anti-farnesyl antibody recognizes both farnesyltransferase and geranyltransferase-induced isoprenylation, GGTase I may selectively geranylgeranylate rather than farnesylate RhoA (34).

Pretreatment of the IAS smooth muscles with simvastatin significantly reduced the levels of prenylated RhoA but caused increase in the levels of unprenylated RhoA, in a concentration-dependent manner (*P < 0.05; n = 4; Fig. 4). Not shown, these effects of simvastatin were reversed by GGPP.

**Effect of HMGCoA reductase inhibitor on the basal smooth muscle tone.** Simvastatin (10 μM) so that the values obtained following the combined treatment were not significantly different from those of controls (*P > 0.05; n = 6; Fig. 1).

**Effect of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitor on percent maximal basal tone of the IAS smooth muscle strips.** Simvastatin (10 μM) so that the values obtained following the combined treatment were not significantly different from those of controls (*P > 0.05; n = 6; Fig. 1).

See Fig. 1 for a diagram of the effect of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitor on percent maximal basal tone of the IAS smooth muscle strips.
separation of corresponding cell homogenates in cytosol and membrane fractions, before and after pretreatments with different concentrations of simvastatin. Data revealed higher levels of RhoA in the IAS membrane vs. the cytosolic fraction (Fig. 5, left). Pretreatment with simvastatin reversed the pattern of distribution, a significant decrease in RhoA in the membrane fraction and increased in the cytosolic fraction, in a concentration-dependent manner (*P < 0.05; n = 4; Fig. 5). These data have been summarized as the ratios of RhoA in particulate/cytosolic expressions (Fig. 5; bottom).

Effect of HMGCoR on the levels of pThr696-MYPTI. To determine specifically the effect of HMGCRI on the ROCK activity, we compared the effects of simvastatin and Y 27632 on pThr696-MYPTI in the IAS. Data show that simvastatin causes concentration-dependent decrease in ROCK activity. The residual levels of pThr696-MYPTI following pretreatment with 10 μM simvastatin and Y 27632 were 28.10 ± 2.56% and 6.35 ± 1.26%, respectively (*P < 0.05; n = 4; Fig. 7). Data suggest that the HMGCRI via RhoA prenylation cause downstream inhibition of ROCK activity, leading to decrease in the IAS tone.

DISCUSSION

The studies for the first time show that the HMG-CoA reductase provides important regulation of the IAS smooth muscle tone via RhoA trafficking regulated by RhoA prenylation. Inhibition of RhoA geranylgeranylation and decrease in the IAS tone with the HMGCRI are similar to that...
with the GGTase I inhibitor GGTI-297. In addition, HMGCR also attenuates the agonist-specific increase in the IAS tone.

It is well known that in the basal state RhoA/ROCK are largely present in the cytosol of the SMCs, and during sustained phase of the smooth muscle contraction they translocate to the membrane during the activation of RhoA/ROCK (36). Activated ROCK inhibits myosin light-chain phosphatase (MLCP) either directly or via phosphorylation of PKC-potentiated inhibitor or endogenous inhibitor of myosin light-chain phosphatase (CPI-17). ROCK-mediated MLCP inhibition decreases dephosphorylation of phosphorylated MLC20 (p-MLC20). The resultant increase in p-MLC20 has been suggested to be responsible for the maintenance of the IAS tone in the basal state (24, 28, 30). There is a large body of evidence for this concept for the agonist-induced sustained contraction of different smooth muscles (16, 21).

Fig. 5. Western blot analyses for the levels of RhoA in the particulate vs. cytosolic fractions of the IAS smooth muscles before (0) and after different concentrations of simvastatin (left), compared with the RSM (right). Densitometric analysis (particulate:cytosolic ratios in relation to α-actin levels) reveal concentration-dependent and significant decrease (*P < 0.05; n = 4) in the levels of RhoA in the particulate fractions of the IAS but not RSM by simvastatin (P > 0.05; n = 4).

Fig. 6. Western blot showing the effect of simvastatin on the levels of Rho kinase (ROCK) II in the cytosolic (C) vs. particulate (P) fractions in the IAS (left) vs. RSM (right). Densitometric analysis (calculated as the particulate:cytosolic ratios) reveals that in the IAS simvastatin and Y 27632 (in comparison with the basal state) cause significant redistribution of ROCK II levels from the particulate to the cytosolic fractions (*P < 0.05; n = 4). However, in the case of RSM, simvastatin or Y 27632 caused no such significant redistributions of ROCK II (P > 0.05; n = 4).
As depicted in Fig. 8, HMG-CoA reductase not only provides the necessary building blocks for cholesterol biosynthesis, but also for the isoprenoids farnesyl and GGPPs (32). These pyrophosphates play a significant role in the posttranslational modification of Ras and Rho GTPase, respectively. HMG-CoA reductase provides upstream regulation of these events. In agreement with this concept, simvastatin, a typical HMGCRI, reduces, not only cholesterol biosynthesis, but also the supply of isoprenoids for Rho prenylation (32). In contrast to the GGTI-297 (which inhibits GGTase I directly), HMGCRI exerts downstream inhibition of RhoA geranylgeranylation via mevalonate/GGPP/GGTase I (2, 37).

Simvastatin, a classical HMGCRI, has been reported to cause vasodilatation of arteries (3). Diminished isoprenoid intermediates are associated with simvastatin-induced decrease in force in vascular smooth muscle from spontaneously hypertensive rats, partially reversible with mevalonate (26). This information is based on the attenuation of agonist-induced smooth muscle contraction, and there are no data in the spontaneously tonic smooth muscle. Our systematic studies in the tonic smooth muscle of the IAS reveal that significant and specific decreases in the basal tone and RhoA/ROCK activity by simvastatin require 24-h incubation.

Longer incubations for the observed changes in the tone and RhoA/ROCK were necessary in the case of, not only HMGCRI, but also RhoA prenylation inhibitor. This is in agreement with the slow turnover of isoprenylation and half life of HMG-CoA reductase (11) lasting hours or days. Following such regimen of pretreatment, simvastatin reduces the basal and agonist-induced increase in the IAS tone. Interestingly, similar results were obtained with the downstream inhibition of geranylgeranylation by GGTI-297 (25). Decrease in IAS tone correlates the decrease in the levels of prenylated RhoA with the corresponding increase in the levels of unprenylated RhoA, in the presence of simvastatin. These findings are of particular interest because similar treatment of the nontonic smooth muscle of the RSM reveals no significant changes either in the force or in the levels of prenylated RhoA.

In agreement with the above concept, we observed significant shifts in the cellular distribution of RhoA/ROCK II following HMGCRI simvastatin, i.e., an increase in the cytosolic and decrease in the membrane RhoA/ROCK II. These data suggest dependence of basal tone on RhoA prenylation, leading to RhoA/ROCK II translocation. This concept draws significant support from the literature as follows. Activation and translocation of RhoA are important processes for the agonist-induced Ca\(^{2+}\) sensitization and force development (14, 36). In this regard, RhoA prenylation appears to be critical (1). Studies by Gong et al. (15) reported that prenylated GTP-RhoA\(^{Val14}\)Ca\(^{2+}\) sensitizes smooth muscle mildly permeabilized with \(\beta\)-escin but not with Triton X-100. These observations suggest that intact membrane is important for RhoA translocation. The studies further demonstrated that unprenylated GTP-RhoA\(^{Val14}\) fails to produce Ca\(^{2+}\) sensitization-dependent changes in smooth muscle contractility.

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Inhibitor Y 27632. This was further evident by the concentration of ROCK II. This is in agreement with the earlier data showing neither simvastatin nor GGTI-297 have any significant effect on the basal tone in the IAS and on biochemical changes in the IAS are reversible with the GGTase inhibitor GGTI-297 cause the redistribution of prenylated RhoA (from the membrane to the cytosol) associated with the decrease in the basal tone in the IAS. These functional and biochemical changes in the IAS are reversible with the GGTase substrate GGPP.

In contrast with the IAS, the data from the RSM reveal that neither simvastatin nor GGTI-297 have any significant effect on the cellular distribution of prenylated RhoA and RhoA/ROCK II. This is in agreement with the earlier data showing predominantly higher levels of GDI bound RhoA (the inhibited form of RhoA) in the phasic smooth muscles (35). In addition, the effect of HMGCR on the basal tone in the IAS and on RhoA/ROCK activation is similar to that of classical ROCK inhibitor Y 27632. This was further evident by the concentration-dependent decrease in ROCK activity following pretreatment of the IAS tissues with simvastatin. These data suggest the significance of sequential events of RhoA prenylation leading to RhoA/ROCK activation.

The inhibitory effect of HMGCR on the spontaneous and thromboxane A2 analog U-46619-induced increase in the IAS tone may be explained on the basis of the endogenous control of the IAS tone by thromboxane pathway, via GPCR activation (8, 9).

In summary, the present studies identify upstream regulation of RhoA prenylation via HMG-CoA reductase as important step in the gastrointestinal smooth muscle tone. These data may be similar to the restorative actions of HMGCRIs in the cardiovascular hypertensive smooth muscles without the untoward effects in normal individuals (38). Consequently, we speculate that HMGCRIs may have significantly more potent inhibitory effects in the hypertensive in contrast with the normotensive IAS. This may explain a lack of adverse gastrointestinal effects of statins in the cardiovascular hypertensive patients on HMGCRI medication. Present findings provide an important mechanism of action of statins in the smooth muscle tone, beyond inhibition of cholesterol biosynthesis.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author.

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