Collagen IV contributes to nitric oxide-induced angiogenesis of lung endothelial cells

Huafang Wang and Yunchao Su

1Department of Pharmacology and Toxicology, 2Department of Medicine, 3Vascular Biology Center, and 4Center for Biotechnology and Genomic Medicine, Medical College of Georgia, Georgia Health Sciences University, Augusta, Georgia

Submitted 8 September 2010; accepted in final form 3 February 2011

Wang H, Su Y. Collagen IV contributes to nitric oxide-induced angiogenesis of lung endothelial cells. Am J Physiol Cell Physiol 300: C979–C988, 2011. First published February 9, 2011; doi:10.1152/ajpcell.00368.2010.—Nitric oxide (NO) mediates endothelial angiogenesis via inducing the expression of integrin αβ3. During angiogenesis, endothelial cells adhere to and migrate into the extracellular matrix through integrins. Collagen IV binds to integrins, leading to integrin activation, which affects a number of signaling processes in endothelial cells. In the present study, we evaluated the role of collagen IV in NO-induced angiogenesis. We found that NO donor 2,2'-hydroxyoxinitrosohydrazino-bis-ethanamine (NOC-18) causes increases in collagen IV mRNA and protein in lung endothelial cells and collagen IV release into the medium. Addition of collagen IV into the coating of endothelial culture increases endothelial monolayer wound repair, proliferation, and tube formation. Inhibition of collagen IV synthesis using gene silencing attenuates NOC-18-induced increases in monolayer wound repair, cell proliferation, and tube formation as well as in the phosphorylation of focal adhesion kinase (FAK). Integrin blocking antibody LM609 prevents NOC-18-induced increase in endothelial monolayer wound repair. Inhibition of protein kinase G (PKG) using the specific PKG inhibitor KT5823 or PKG small interfering RNA prevents NOC-18-induced increases in collagen IV protein and mRNA and endothelial angiogenesis. Together, these results indicate that NO promotes collagen IV synthesis via a PKG signaling pathway and that the increase in collagen IV synthesis contributes to NO-induced angiogenesis of lung endothelial cells through integrin-FAK signaling. Manipulation of collagen IV could be a novel approach for the prevention and treatment of diseases such as alveolar capillary dysplasia, severe pulmonary arterial hypertension, and tumor invasion.

extracellular matrix; NOC-18; angiogenic; endothelium

ANGIOGENESIS PLAYS AN ESSENTIAL role in a number of physiological and pathological processes such as lung alveolus development (31), proliferative retinopathy (1), carcinogenesis (24), and lung tissue repair (10, 17). The process of angiogenesis involves endothelial cell migration, proliferation, and differentiation as well as tube formation (4, 5, 18). Nitric oxide (NO) has been shown to play an important role in the regulation of angiogenesis. For example, growth factor-induced formation of angiillary-like structures of human umbilical venous endothelial cells is blocked by NO synthase antagonist (27). A cornea model of angiogenesis induced by VEGF is antagonized by nitro-L-arginine methyl ester (37). Additionally, angiogenesis is impaired in mice lacking the gene for endothelial NO synthase (21). Studies have shown that NO directly contributes to endothelial cell migration, proliferation, and capillary tube formation (2, 22). It is well documented that NO targets and activates guanylyl cyclase, resulting in increased cGMP levels, which are responsible for the vasodilation and anticoagulation actions of NO (20). However, the mechanisms by which NO induces angiogenesis are not fully understood. It is well documented that cGMP activates its downstream signal PKG, which is responsible for a number of NO/cGMP-mediated processes such as barrier protection and angiogenesis (19, 30). Senthilkumar et al. (30) reported that sildenafil, a phosphodiesterase inhibitor, promotes angiogenesis through a PKG-dependent pathway. Nevertheless, the detail mechanism for PKG-mediated angiogenesis remains unknown.

It has been reported that NO regulates angiogenesis via an integrin-dependent mechanism (22). Lee et al. (16) found that NO induces the expression of integrin αβ3 in endothelial cells supporting endothelial cell migration, survival, and angiogenesis. During angiogenesis, endothelial cells adhere to and migrate into the extracellular matrix through integrins. Binding of integrin αβ3 to collagen IV affects endothelial cell adhesion, migration, and proliferation in the process of angiogenesis (25, 28). Collagen IV, a major component of the basement membrane underlying endothelium, is the major type of collagen synthesized and secreted by vascular endothelial cells (9). Inhibition of collagen IV synthesis using cis-hydroxyproline blocked tube formation, whereas the addition of exogenous collagen IV to the matrigel increased tube formation (6). These data suggest that collagen IV is an important regulator of endothelial angiogenesis. However, it is not clear whether and how collagen IV plays a role in NO-induced angiogenesis. In the present study, we found for the first time that NO induces collagen IV synthesis from vascular endothelial cells via a PKG signaling pathway. Inhibition of collagen IV synthesis using gene silencing attenuates NO-induced endothelial angiogenesis and phosphorylation of focal adhesion kinase (FAK), indicating that the PKG-mediated synthesis of collagen IV contributes to the proangiogenic effect of NO on vascular endothelial cells.

MATERIALS AND METHODS

Reagents. Collagen IV, selective PKG inhibitor KT5823, PDGF-BB, and bovine serum albumin (BSA) were purchased from Sigma-Aldrich (St Louis, MO). 2,2'-Hydroxyoxinitrosohydrazino-bis-ethanamine (NOC-18) was purchased from Calbiochem (San Diego, CA). Rabbit anti-collagen IV A2 antibody and goat anti-PKGα were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The antibodies against total FAK and phospho-FAK (pTyr597) were from Cell Signaling Technology (Danvers, MA). Mouse anti-integrin αβ3, LM609 was from Millipore (Temecula, CA). Matrigel was from BD Biosciences Discovery Labware (Bedford, MA).

Cell culture. Pulmonary artery endothelial cells (PAEC) and aortic endothelial cells were obtained from the main pulmonary artery and...
thoracic aorta of 6- to 7-mo-old pigs at a local abattoir. Third to sixth passage cells in monolayer culture were maintained in RPMI 1640 medium containing 10% FBS and antibiotics (10 U/ml penicillin, 100 
μg/ml streptomycin, 20 μg/ml gentamicin, and 2 μg/ml Fungizone) as described previously (15) and were used 2 or 3 days after confluence.

Determination of angiogenesis in vitro. Angiogenesis was evaluated in vitro by measuring migration, proliferation, and tube formation as previously reported by us (32). Endothelial migration was evaluated by measuring endothelial monolayer wound repair. Briefly, a cell-free wound zone was created by scraping the monolayer with a sterile pipette tip. The wound width of monolayers in millimeters was measured under the microscope. Then monolayers were washed and incubated in 5% CO2 at 37°C. Wound width was measured again after an additional 16 h of incubation. Endothelial monolayer wound repair distance was expressed as the width of the wound before treatment subtracted by that after treatment. PAEC proliferation was assayed with a kit from Roche (Indianapolis, IN) that monitors incorporation of 5-bromo-2′-deoxyuridine (BrdU) into newly synthesized DNA. PAEC were seeded into 96-well plates (5 × 103 cells/well). After treatment, the newly synthesized DNA was labeled by incubating cells with BrdU (10 μM) for 8 h. The BrdU was detected by use of anti-BrdU-peroxidase conjugate as described in the manufacturer’s instruction. Absorbance at 450 nm was measured using a SpectraMax M2e microplate reader (Molecular Devices, Sunnyvale, CA). To do the tube formation assay, 96-well culture plates were coated with 50 
μl of matrigel (BD Biosciences Discovery Labware) per well, then allowed to polymerize for 30 min at 37°C. PAEC were seeded on coated plates at a density of 2 × 104 cells/well in RPMI 1640 medium containing 1% FBS at 37°C. The images of tubes in each well were taken at 8 h with a digital output camera (Olympus) attached to an inverted phase-contrast microscope at ×100 magnification. The tube length was measured with AxioVision LE software (Carl Zeiss Imaging Solutions) and is expressed as pixel units.

Western blot analysis. After treatments, PAEC were washed with phosphate-buffered saline (PBS) and lysed in boiled sample buffer (0.06 M Tris-HCl, 2% SDS, and 5% glycerol, pH 6.8). The lysates were boiled for 5 min. The lysate proteins (20 to 40 μg) were separated on a 4–20% Tris-glycine SDS-PAGE and electrophoretically transferred onto nitrocellulose membranes. The membranes were incubated in blocking solution at room temperature for 1–2 h and then hybridized with primary antibody against collagen IV, PKG1α, total FAK, pTyr397-FAK, or α,β3-integrin overnight at 4°C. The bands were detected by an immunochemiluminescence method. The density was quantitated by Bio-Rad Quantity One Software.

Determination of collagen IV mRNA (COL 4A3). After treatment, total RNA of PAEC was extracted by using an RNAeasy Mini Kit from Qiagen. To measure COL 4A3 mRNA content, quantitative real-time RT-PCR was performed by using TaqMan gene expression assay from Applied Biosystems (assay ID Ss03374310_u1; Foster City, CA). The primer sequence was not disclosed by the company. ABI 7500 Sequence Detector (Applied Biosystems) was programmed for the PCR conditions as follows: 95°C for 10 min, 40 cycles of 95°C for 15 s and 60°C for 1 min. COL 4A3 mRNA content was expressed as 2−ΔΔCT using 18S RNA as a reference.

Knockdown of collagen IV and PKG expression using the small interfering RNA strategy. The expressions of collagen IV and PKG were silenced by using small interfering RNA (siRNA) technology. The siRNAs were obtained from Santa Cruz Biotechnology (sc-72954 for collagen IV siRNA and sc-35059 for PKG siRNA). A negative control siRNA (no. AM4611, Applied Biosystems) was used as control. The sequences of these siRNAs are not disclosed by the companies. The siRNAs were transfected into PAEC using Qiagen RNAiFect transfection reagent according to the manufacturer’s instructions. After a 48-h incubation, the cells were subjected to the measurements of endothelial monolayer wound repair, tube formation, and proliferation.

Statistical analysis. In each experiment, experimental and control endothelial cells were matched for cell line, age, seeding density, number of passages, and number of days postconfluence to avoid variation in tissue culture factors that can influence measurements of angiogenesis and collagen IV level. Results are shown as means ± SE for n experiments. One-way ANOVA and post-t-test analyses were used to determine the significance of differences between the means of different groups. P < 0.05 was considered statistically significant.

RESULTS

NO donor NOC-18 increases collagen IV synthesis in endothelial cells. There are at least 27 types of human collagens which are numbered by the order of discovery (23). Collagen I, II, III, and IV are the major types of collagen in mammalian tissues. We measured the mRNAs of collagen I, II, III, and IV in lung endothelial cells using quantitative real-time PCR and found that the amounts of mRNAs of collagen I, II, and III
were too low to be detected. However, lung endothelial cells contain abundant collagen IV mRNA and protein. Thus, we studied the effect of NO donor NOC-18 on collagen IV mRNA and protein in PAEC. We found that incubation of PAEC with NOC-18 in concentrations of 1–100 μM caused a dose-dependent increase in collagen IV mRNA in PAEC (Fig. 1A). The collagen IV mRNA reached plateau at NOC-18 concentration of 10 μM. Moreover, the increase in intracellular collagen IV protein content reached the maximum at NOC-18 concentration of 10 μM and returned to control level at 100 μM (Fig. 1, B and C). However, the collagen IV protein content in the culture medium increased and remained high at NOC-18 concentration of 100 μM, suggesting that NOC-18 in higher concentrations may increase collagen IV release from endothelial cells. NOC-18 has a half-life of 20 h in physiological pH at 37°C. The actual NO concentration of 100 μM of NOC-18 is 673 nM (8). Because the physiological concentration of NO is around 100 nM (8, 14), the applied dose of NOC-18 here is relevant to physiological NO concentration.

Collagen IV enhances monolayer wound repair, proliferation, and tube formation of PAEC. Endothelial monolayer wound repair and proliferation were measured on plates coated with collagen IV or BSA. We found that collagen IV significantly increased PAEC monolayer wound repair, proliferation, and tube formation (Fig. 2). However, BSA in the same dose had no effect on angiogenesis, suggesting that the increases in monolayer wound repair, proliferation, and tube formation were the specific effects of collagen IV on endothelial cells.

Knockdown of collagen IV inhibits NOC-18-induced increase in monolayer wound repair, proliferation, and tube formation of PAEC. To study whether the increase in collagen IV contributes to NOC-18-induced angiogenesis, collagen IV gene was silenced by siRNA strategy. As shown in Fig. 3, A and B, siRNA directed against collagen IV mRNA significantly reduced collagen IV protein expression and prevented NOC-18-induced increases in collagen IV protein content in the cells and culture medium. Knockdown of collagen IV also decreased endothelial monolayer wound repair, cell proliferation,
and tube formation and attenuated NOC-18-induced increases in endothelial monolayer wound repair, cell proliferation, and tube formation (Fig. 3, C–F). These results suggest that synthesis of collagen IV plays an important role in NOC-18-induced angiogenesis.

PKG inhibitor KT5823 attenuates NOC-18-induced increase in collagen IV, integrin αvβ3, and angiogenesis. To determine whether a cGMP/PKG signaling pathway is involved in NOC-18-induced increases in collagen IV synthesis and angiogenesis, PAEC were incubated with NOC-18 in

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Fig. 4. Specific PKG inhibitor KT5823 prevents NOC-18-induced increases in collagen IV synthesis, endothelial monolayer wound repair, cell proliferation, and tube formation. Intracellular collagen IV protein content (A and B), collagen IV mRNA (C), monolayer wound repair (D), cell proliferation (E), and tube formation (F and G) were evaluated in control PAEC and PAEC exposed to NOC-18 (10 μM) in the absence and presence of KT5823 (10 μM). A: representative immunoblot of collagen IV protein from 6 experiments. B: bar graph depicting the changes in intracellular collagen IV protein. C: bar graph showing the changes in tube length. Results are expressed as means ± SE; n = 5 or 6 experiments. *P < 0.05 vs. vehicle group in control.
the absence and presence of a specific PKG inhibitor, KT5823 (3). Then collagen IV mRNA and protein content, monolayer wound repair, proliferation, and tube formation of PAEC were measured. As shown in Fig. 4, A–C, incubation of PAEC with KT5823 (10 μM) decreased collagen IV mRNA level and collagen IV protein content in the cells and culture medium. KT5823 also inhibited NOC-18-induced increases in collagen IV mRNA level and collagen IV protein content in the cells and culture medium. Moreover, KT5823 treatment inhibited endothelial wound repair, cell proliferation, and tube formation and attenuated NOC-18-induced increases in endothelial monolayer wound repair, cell proliferation, and tube formation (Fig. 4, D–G). In addition, KT5823 prevented NOC-18-induced increase in integrin αvβ3 protein (Supplemental Fig. S1; Supplemental Material for this article is available online at the Journal website). These data indicate that a cGMP/PKG signaling pathway is involved in NOC-18-induced increases in collagen IV synthesis and angiogenesis.

Fig. 5. Knockdown of PKG prevents NOC-18-induced increase in angiogenesis. PAEC were transfected with control siRNA or siRNA against the mRNA of PKG. After 48 h, the cells were incubated with or without NOC-18 (10 μM) for 16 h and then intracellular protein content of PKG and collagen IV (A and B) and collagen IV mRNA (C) was assayed. The monolayer wound repair (D), cell proliferation (E), and tube formation (F and G) with or without NOC-18 (10 μM) were measured as described in MATERIALS AND METHODS. A: representative immunoblot of collagen IV and PKG protein from 5 experiments. B: bar graph depicting the changes in intracellular protein content of PKG and collagen IV. F: representative images from 5 tube formation assays (amplification, ×100). G: bar graph showing the changes in tube length. Results are expressed as means ± SE; n = 5 experiments. *P < 0.05 vs. vehicle group (without NOC-18); #P < 0.05 vs. vehicle group with control siRNA.
Knockdown of PKG inhibits NOC-18-induced increase in collagen IV and angiogenesis. To further confirm whether NOC-18-induced increases in collagen IV synthesis and angiogenesis occur through a PKG signaling pathway, PKG gene expression was silenced by siRNA treatment. As shown in Fig. 5, A–C, knockdown of PKG protein decreased collagen IV mRNA and protein levels and prevented NOC-18-induced increases in levels of collagen IV mRNA and collagen IV protein in the cells and culture medium. More importantly, knockdown of PKG protein inhibited endothelial monolayer wound repair, cell proliferation, and tube formation and suppressed the increases in endothelial monolayer wound repair, cell proliferation, and tube formation induced by NOC-18 (Fig. 5, D–G). Together, these results indicate that PKG signaling is responsible for NO-induced collagen IV synthesis and angiogenesis.

Knockdown of collagen IV inhibits NOC-18-induced increase in the phosphorylation of FAK. Binding of collagen IV to integrin α5β3 of endothelial cells leads to integrin activation which affects a number of signaling processes in endothelial cells (25, 28). Integrin activation initiates FAK signaling and facilitates cell adhesion, migration, proliferation, and differentiation (13). To determine whether the role of collagen IV in NO-induced angiogenesis involves FAK signaling, phosphorylation of FAK was measured in PAEC in which collagen IV was knocked down. As shown in Fig. 6, incubation of PAEC with NOC-18 for 16 h caused an increase in FAK phosphorylation. Knockdown of collagen IV decreased FAK phosphorylation and inhibited the NOC-18-induced increase in FAK phosphorylation (Fig. 6), suggesting that collagen IV is required for maintaining the phosphorylation status of FAK and that the collagen IV-mediated proangiogenic effect of NO occurs through FAK signaling.

Integrin blocking antibody LM609 prevents NOC-18-induced increase in endothelial monolayer wound repair. To further prove that NOC-18-induced angiogenesis response is indeed caused by binding of extracellular collagen IV to
integrin $\alpha_3\beta_3$ and not by an unresolved intracellular signaling effect, integrin $\alpha_3\beta_3$ was blocked using a blocking antibody LM609. As shown in Fig. 7, the NOC-18-induced increase in endothelial monolayer wound repair was prevented by antibody LM609, suggesting that the collagen IV-mediated proangiogenic effect of NO is truly through integrin-FAK signaling.

**Fig. 6.** Knockdown of collagen IV attenuates NOC-18-induced increase in focal adhesion kinase (FAK) phosphorylation. PAEC were transfected with control siRNA or siRNA against collagen IV mRNA. After 48 h, protein content of phosphorylated FAK (pTyr397) and total FAK were measured as described in MATERIALS AND METHODS. A: representative immunoblot of phosphorylated FAK and total FAK from 4 experiments. B: bar graph depicting the changes in protein content of phosphorylated FAK and total FAK. Results are expressed as means ± SE; $n = 4$ experiments. *$P < 0.05$ vs. vehicle group (without NOC-18); #$P < 0.05$ vs. vehicle group with control siRNA.

**Fig. 7.** Integrin $\alpha_3\beta_3$ blocking antibody LM609 prevents NOC-18-induced increase in endothelial monolayer wound repair. PAEC were incubated with integrin $\alpha_3\beta_3$ blocking antibody LM609 (10 $\mu$g/ml) or nonimmune mouse IgG (10 $\mu$g/ml) for 16 h, after which endothelial monolayer wound repair was evaluated. Results are expressed as means ± SE; $n = 4$ experiments. *$P < 0.05$ vs. vehicle group.

**Fig. 8.** A schematic pathway illustrating the role of collagen IV and PKG in nitric oxide (NO)-induced angiogenesis. NO activates PKG and induces the synthesis of collagen IV and integrin $\alpha_3\beta_3$. Collagen IV in the extracellular matrix (ECM) binds to integrin $\alpha_3\beta_3$, leading to the increase in FAK phosphorylation in the focal adhesion and angiogenesis.

**DISCUSSION**

The major new finding of the present study is that NO induces collagen IV synthesis from lung vascular endothelial cells. The increases in mRNA and protein content of collagen IV are not due to increases in the amount of cells caused by increased proliferation, because these results have been normalized to internal control levels of GAPDH and 18S rRNA. Additionally, inhibition of collagen IV synthesis using gene silencing attenuates endothelial monolayer wound repair, cell proliferation, and tube formation induced by the NO donor NOC-18. Addition of collagen IV into the coating of endothelial cell culture increases endothelial monolayer wound repair, proliferation, and tube formation. These data represent the first evidence that increased synthesis of collagen IV contributes to NO-induced angiogenesis of lung endothelial cells (Fig. 8).

There are at least 27 types of human collagens which have been numbered by the order of discovery (23). Collagen I, II, III, and IV are the major types of collagen in mammalian tissues. We found that the amounts of mRNA of collagen I, II, and III in endothelial cells were too low to be detected. However, our experiments showed that lung endothelial cells contain abundant collagen IV mRNA and protein, which confirms a prior report for vascular endothelial cells (9). Unlike other types of collagen, collagen IV is an exclusive component of the basement membranes. Through complex inter- and intramolecular interactions it forms complex networks. Our data indicate that NO induces synthesis and release of collagen IV from lung endothelial cells. Binding of collagen IV to integrin $\alpha_3\beta_3$ of endothelial cells leads to integrin activation which affects a number of signaling processes in endothelial cells (25, 28). Integrin activation has been shown to initiate FAK signaling and facilitate cell adhesion, migration, proliferation, and differentiation (13). Collagen IV-induced integrin activation also protects pulmonary vascular endothelial cells against apoptosis upon exposure to bleomycin and LPS (11, 12). In our study, inhibition of collagen IV synthesis by siRNA strategy attenuates NOC-18-induced increases in endothelial wound repair, cell proliferation, and tube formation. These findings indicate that the increase in collagen IV synthesis contributes to NO-induced angiogenesis. In addition, we found that inhibition of collagen IV synthesis decreases FAK phos-
phorylation and inhibits NO-induced increase in FAK phosphorylation. Furthermore, NOC-18-induced increase in monolayer wound repair can be prevented by integrin blocking antibody. Together, these data suggest that collagen IV contributes to the proangiogenic effect of NO through integrin-FAK signaling.

We have previously reported that a specific inhibitor of the soluble guanylyl cyclase, 1H-[1,2,4]oxadiazolo[4,3-a]quinoxaline-1-one (ODQ), prevents the angiogenic effect of NOC-18 and S-nitroso-N-acetyl-DL-penicillamine (SNAP), suggesting that NO-induced angiogenesis is via a cGMP-dependent mechanism (33). cGMP activates PKG which is responsible for a number of NO/cGMP-mediated processes such as barrier protection and angiogenesis (19, 30). The results obtained in the present study show that the specific PKG inhibitor KT5823 prevents increases in collagen IV protein and mRNA levels and in endothelial wound repair, cell proliferation, and tube formation induced by NOC-18. Furthermore, by use of specific siRNA, we have demonstrated that knockdown of the protein expression of PKG inhibits increases in collagen IV synthesis and angiogenesis induced by NOC-18. Additionally, KT5823 prevents NOC-18-induced increase in integrin α3β3. Taken together, these results indicate that NO-induced collagen IV and integrin α3β3 synthesis and angiogenesis requires PKG activation (Fig. 8).

We also documented that NOC-18 induces collagen IV synthesis in aortic endothelial cells (Supplemental Fig. S2), indicating that NO-induced increase in collagen IV synthesis is not specific in PAEC. Notably, knockdown of collagen IV and inhibition of PKG not only blocked NOC-18-stimulated responses, but also reduced basal proliferation, wound repair, and tube formation. Nevertheless, knockdown of collagen IV does not inhibit the increase in endothelial monolayer repair induced by PDGF-BB (Supplemental Fig. S3) which does not stimulate NO production in endothelial cells (36). Thus, collagen IV is not only a necessary prerequisite for angiogenesis but also directly contributes to the specific NO-stimulated angiogenic response.

Abnormality of lung angiogenesis is implicated in the pathogenesis of several pulmonary diseases. For example, endothelial NO synthase knockout mice develop alveolar capillary dysplasia in lung alveolus development (7). Moreover, the plexiform lesion formed in severe pulmonary arterial hypertension (PAH) contains large amounts of collagen IV (34). In view of the importance of NO in angiogenesis and shear stress-induced signaling, our novel discovery that NO-induced collagen IV synthesis contributes to angiogenesis of lung endothelial cells has therapeutic potential. Manipulation of collagen IV could be a novel approach for the prevention and treatment of angiogenesis-related diseases such as abnormal pulmonary alveolarization, severe pulmonary arterial hypertension, proliferative retinopathy, and tumor invasion.

ACKNOWLEDGMENTS

We thank Dr. William R. Caldwell for critical reading of the manuscript.

GRANTS

This work was supported by National Institutes of Health Grant R01HL088261, Flight Attendants Medical Research Institute Grant 072104, and American Heart Association Greater Southeast Affiliate Grant 0855338E.

DISCLOSURES

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

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