Sustained adenosine exposure causes lung endothelial apoptosis: a possible contributor to cigarette smoke-induced endothelial apoptosis and lung injury

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Lu Q, Sakhatksky P, Newton J, Shamirian P, Hsiao V, Curren S, Gabino Miranda G, Pedroza M, Blackburn MR, Rounds S. Sustained adenosine exposure causes lung endothelial apoptosis: a possible contributor to cigarette smoke-induced endothelial apoptosis and lung injury. Am J Physiol Lung Cell Mol Physiol 304:L361–L370, 2013. First published January 11, 2013; doi:10.1152/ajplung.00161.2012.—Pulmonary endothelial cell (EC) apoptosis has been implicated in the pathogenesis of emphysema. Cigarette smoke (CS) causes lung EC apoptosis and emphysema. In this study, we show that CS exposure increased lung tissue adenosine levels in mice, an effect associated with increased lung EC apoptosis and the development of emphysema. Adenosine has a protective effect against apoptosis via adenosine receptor-mediated signaling. However, sustained elevated adenosine increases alveolar cell apoptosis in adenosine deaminase-deficient mice. We established an in vitro model of sustained adenosine exposure by incubating lung EC with adenosine in the presence of an adenosine deaminase inhibitor, deoxycoformicin. We demonstrated that sustained adenosine exposure caused lung EC apoptosis via nucleoside transporter-facilitated intracellular adenosine uptake, subsequent activation of p38 and JNK in mitochondria, and ultimately mitochondrial defects and activation of the mitochondria-mediated intrinsic pathway of apoptosis. Our results suggest that sustained elevated adenosine may contribute to CS-induced lung EC apoptosis and emphysema. Our data also reconcile the paradoxical effects of adenosine on apoptosis, demonstrating that prolonged exposure causes apoptosis via nucleoside transporter-mediated intracellular adenosine signaling, whereas acute exposure protects against apoptosis via activation of adenosine receptors. Inhibition of adenosine uptake may become a new therapeutic target in treatment of CS-induced lung diseases.

mitochondria; nucleoside transporters; oxidative stress; adenosine; cigarette smoke

INCREASED ENDOTHELIAL CELL (EC) apoptosis has been observed in humans and animals with emphysema (31, 32), acute lung injury (ALI) (1, 25), idiopathic pulmonary fibrosis (IPF) (13, 34, 64), and ischemia-reperfusion (I/R) lung injury (48, 51). Inhibition of alveolar cell apoptosis attenuates the emphysematous changes (32, 50, 61), increases survival rate in ALI (33), and enhances the function of transplanted lungs (51) in animal models. These results indicate that excessive lung EC apoptosis plays a role in pathogenesis of some lung diseases (26, 40).

The purine nucleoside adenosine (Ado) is a potent signaling molecule. Extracellular Ado exists in low concentrations (40–600 nM) under homeostatic conditions and is elevated by platelet degranulation and cell necrosis. In response to inflammation and tissue injury, extracellular Ado levels are also increased due to increased expression and/or activation of ectonucleotidases (CD39 and CD73) and/or decreased expression and/or activation of Ado deaminase (ADA) (21, 22, 63, 66). It has been reported that Ado levels are significantly elevated in plasma of humans with sepsis-induced ALI (43, 60) and tissue ischemia (59). Ado levels are also increased in bronchoalveolar lavage (BAL) fluid of human smokers (20). However, it is unknown whether lung tissue Ado levels are altered in smokers or patients with cigarette smoke (CS)-associated lung diseases. It is well documented that CS is a major risk factor for emphysema. CS also increases susceptibility to ALI (10). Therefore, understanding the effect of CS on lung Ado levels and the role of sustained elevated Ado on lung EC apoptosis may provide novel insights into the mechanisms by which CS predisposes to a variety of lung diseases.

Ado achieves its physiological and pathological roles via either activation of G protein-coupled Ado receptors (AR), including A1R, A2AR, A2BR, and A3R, or nucleoside transporter (NT)-facilitated intracellular Ado uptake and metabolism. Activation of AR-mediated Ado signaling has been shown to be protective against apoptosis and tissue injury in multiple organs. For example, activation of A2AR (54) and A1R (14, 44, 55) reduces I/R brain and lung injury and apoptosis. CD39 transgenic mice are protected against renal I/R vascular injury and apoptosis, and these beneficial effects are attributed to increased extracellular Ado and subsequent activation of A2BR (17). Activation of A1R also blunts staurosporine-induced apoptosis of astrocytes (18). Additionally, activation of A2BR protects against apoptosis of remote cardiac myocytes in an infarcted heart (57).

In contrast to the protective effect of AR-mediated signaling, the stable Ado analog, 2-chloroadenosine, causes apoptosis and increases oxidative stress in prostate cancer cells (46). Studies of ADA-deficient mice indicate that sustained elevation of Ado caused chronic lung injury and alveolar cell apoptosis, effects that were exacerbated by deletion of A2BR (69). The mechanism by which sustained exposure to elevated Ado causes lung EC apoptosis is not known.

Since Ado is rapidly metabolized by ADA, we developed a model of sustained Ado exposure by incubating lung EC with Ado in the presence of an ADA inhibitor, deoxycoformicin (DCF) (38). We found that sustained Ado exposure caused lung EC apoptosis via NT-mediated intracellular events. We further showed that sustained elevated Ado after uptake caused activation of p38 and JNK, subsequent mitochondrial defects,
and ultimately activation of the mitochondria-mediated intrinsic pathway of apoptosis. We also documented that lung tissue Ado levels were elevated in AKR mice exposed to CS for 3 wk, an effect associated with increased lung EC apoptosis and the development of emphysema. These results suggest that sustained elevated Ado in the lungs may contribute to CS-induced lung EC apoptosis and tissue injury.

MATERIALS AND METHODS

Cells and reagents. Bovine pulmonary artery ECs were purchased from Vec Technologies (Rensselaer, NY) and were used between passages 3 and 9. The pulmonary artery ECs were propagated in MEM medium containing 10% fetal bovine serum and sodium pyruvate. Ado, dipyridamole (DPM), nitrobenzylthiocyanate (NBTC), DADME, N7(4-cyanophenylcarbamoylmethyl)xanthine (DPCPX), N7-di(4-(4-cyanophenylcarbamoylmethyl)xanthine (DPMX), and 8-[(4-cyanophenylcarbamoylmethyl)oxy]enephyll-1,3-di-((R)-2-methyl-6-phenylethynyl-1,4-(/H11006)tained from Cell Signaling Technology (Danvers, MA). Antibodies directed against caspase-3, Bax, and caspase-8 were obtained from Cell Signaling Technology (Danvers, MA). Antibodies directed against caspase-9 and Bad were purchased from Stressgen (San Diego, CA). Antibodies directed against Bcl-2 were purchased from BD Biosciences (San Jose, CA). Antibodies directed against actin and GAPDH were from Santa Cruz (Santa Cruz, CA). Antibody against von Willebrand factor (vWF) was from DAKO (Carpinteria, CA). IC-1 was from Cayman Chemical (Ann Arbor, MI). 4,6-Diamidino-2-phenylindole (DAPI) (Prolong Gold antifade reagent with DAPI) and MitoTracker Red CMXRos were from Invitrogen (Grand Island, NY). ApopTag peroxidase In Situ Apoptosis Detection Kit [terminal transferase-mediated dUTP nick end-labeling (TUNEL) kit] was purchased from EMD Millipore (Billerica, MA). DAB and VIP peroxidase substrates kits were from Vector Laboratories (Burlingame, CA).

CS exposure of mice. Animal protocols were approved by the Providence Veterans Affairs Medical Center Institutional Animal Care and Use Committee and complied with the Health Research Extension Act and the Public Health Service policy. C57BL/6J and AKR mice were exposed to CS for 6 h/day, 4 days/wk, for 3 wk using a TE-10 mouse smoking machine (Teague Enterprises, Woodland, CA) and 3R4F reference cigarettes (University of Kentucky, Tobacco Research Institute, Lexington, KY), as described (52). Each cigarette was puffed for 2 s, once every minute for a total of eight puffs, at a flow rate of 1.05 l/min, to provide a standard puff of 35 cm³. The smoke machine was adjusted to produce a mixture of sidestream
studies, three to six mice per group were used. Means and SE were calculated based on the values of each animal in each group and the numbers of animals used in that group. Data are presented as means ± SE. The difference between two means was assessed using Student’s t-test, and the differences among three or more means were assessed using ANOVA followed by Tukey-Kramer post hoc test. Differences among means are considered significant when \( P < 0.05 \).

RESULTS

CS-induced increase in lung Ado levels was associated with lung EC apoptosis and early onset of emphysema. CS exposure causes lung EC apoptosis and emphysema in humans (31). Ado levels are increased in BAL fluid of human smokers (20). Whether CS increases lung tissue Ado is unknown. We examined the effects of CS on lung tissue Ado in C57BL/6J mice, which are mildly susceptible to CS-induced emphysema, and in AKR mice, which are highly susceptible to CS-induced emphysema (27). We found that lung tissue Ado levels were significantly elevated in AKR mice exposed to CS for 3 wk, but not in C57BL/6J mice exposed to CS for either 6 h or 3 wk (Fig. 1A). As expected, after 3 wk of CS exposure, C57BL/6J mice did not show any sign of emphysema, with no change in volume loops obtained following manufacturer’s recommendations. Among means are considered significant when \( P < 0.05 \).
either static lung compliance (Fig. 1B) or mean linear intercept (data not shown). In contrast, AKR mice displayed a trend of increased mean linear intercept (data not shown) and a significant increase in static lung compliance (Fig. 1B), indicating the onset of early emphysema. Temporally correlating with increased lung Ado and static lung compliance, AKR mice also exhibited increased lung EC apoptosis after 3 wk of CS exposure (Fig. 1, C and D). However, increased lung EC apoptosis was not seen in C57BL/6J mice exposed to CS for 3 wk (data not shown). Thus our data demonstrate a strong association among lung tissue Ado levels, lung endothelial apoptosis, and emphysema upon 3 wk of CS exposure. Similar to a previous report by others (28), we also noticed that the baseline levels of static lung compliance of AKR mice were significantly lower than that of C57BL/6J mice (Fig. 1B).

Sustained Ado exposure caused lung EC apoptosis. Sustained elevated Ado in ADA-deficient mice causes alveolar cell apoptosis (69). To determine whether sustained exposure to elevated Ado causes lung EC apoptosis, we used our previously established in vitro model of sustained Ado exposure (38) by incubating lung EC with Ado plus ADA inhibitor, DCF, for up to 48 h. Because Ado can be rapidly metabolized by ADA, EC exposed to Ado plus DCF maintained high concentrations of intracellular Ado for up to 48 h, whereas EC exposed to vehicle, Ado, or DCF alone had very low levels of Ado (38). Similar to the in vivo effect of sustained Ado on alveolar cell apoptosis (69), lung EC exposed to elevated Ado for 24 and 48 h, but not 5 h, had an increased cleaved (active) caspase-3 (Fig. 2A). The effect of sustained elevated Ado on lung EC apoptosis was also demonstrated by increased chro-

![Image](http://ajplung.physiology.org/)
matin condensation in apoptotic nuclei by DAPI staining (Fig. 2B) and by elevated TUNEL-positive cells (Fig. 3C) in cultures exposed to Ado plus DCF for 24 h.

Sustained Ado exposure caused lung EC apoptosis via NT-mediated intracellular uptake. We have previously shown that sustained Ado exposure causes lung endothelial barrier dysfunction via NT-mediated intracellular Ado uptake (38). Similarly, inhibition of NTs with DPM completely prevented sustained Ado exposure-induced caspase-3 cleavage (Fig. 3A) and apoptosis (Fig. 3, B and C). A similar protective effect was seen when another NT inhibitor, NBTI, was used (Fig. 3A). However, antagonists for A1R (DPCPX), A2AR (DPMX), A2BR (MRS1754), and A3R (MRS1191) did not protect, but instead exacerbated, sustained Ado exposure-induced caspase-3 activation (Fig. 3D). These results indicate that sustained Ado exposure causes lung EC apoptosis via NT-mediated intracellular events, and that AR-mediated signaling may limit EC apoptosis.

Activation of p38 and JNK mediated sustained Ado exposure-induced EC apoptosis and mitochondrial defects. Our laboratory has previously shown that sustained Ado exposure increased oxidative stress and activated the redox-sensitive proteins, p38 and JNK, via NT-mediated intracellular Ado uptake (38). To our surprise, neither antioxidant, NAC, nor NADPH oxidase inhibitor, apocynin, altered sustained Ado-induced caspase-3 cleavage (Fig. 4A). However, inhibition of p38 by SB203580 and inhibition of JNK by SP600125 blunted sustained Ado-induced caspase-3 cleavage (Fig. 4B) and apoptosis (Fig. 4C).

Our laboratory has previously shown that activation of p38 and JNK by sustained Ado exposure occurs in mitochondria (38). Thus we hypothesized that sustained Ado exposure caused EC apoptosis via p38- and JNK-mediated mitochondrial defects. We first assessed mitochondria morphology and found that mitochondria in normal lung EC form an intricate filamentous network, as stained by MitoTracker Red CMXRos.
NT-mediated activation of p38 and JNK causes lung endothelial apoptosis and mitochondria defects via SB203580 (Fig. 4E, DPM, SP600125, and DCF). Sustained Ado exposure disrupted the network, resulting in dysmorphic mitochondria (Fig. 4D, A+D). A similar structural mitochondrial defect has been linked to mitochondrial dysfunction (9). Interestingly, inhibition of p38 with SB203580 abolished sustained Ado-induced changes in mitochondrial morphology (Fig. 4D, SB+A+D). A similar protective effect was seen when JNK inhibitor, SP600125, was used (data not shown).

The loss of mitochondrial membrane potential is a hallmark for apoptosis. To further examine if sustained Ado exposure decreases mitochondrial membrane potential, we assessed the loss of mitochondrial membrane potential by JC-1 staining. In healthy cells, JC-1 exists as a monomer in the cytosol (green) and also accumulates as red aggregates in the mitochondria. In apoptotic cells, JC-1 exists in monomeric (green) form in cytosol, without red mitochondrial aggregates. Lung EC treated with vehicle for 48 h displayed numerous red aggregates (Fig. 4E, V), indicating intact mitochondrial membrane potential. These red aggregates were much less evident in EC treated with Ado plus DCF for 48 h (Fig. 4E, A+D), indicating loss of mitochondrial membrane potential. Sustained Ado-induced loss of mitochondrial membrane potential was abolished by NT inhibitor, DPM, JNK inhibitor, SP600125, and p38 inhibitor, SB203580 (Fig. 4E, DPM+A+D, SP+A+D, SB+A+D). These results suggest that sustained Ado exposure causes lung endothelial apoptosis and mitochondria defects via NT-mediated activation of p38 and JNK.

**Sustained Ado exposure caused EC apoptosis via activation of mitochondria-mediated apoptotic pathway.** Our laboratory has previously shown that p38 and JNK were activated in mitochondria by sustained Ado exposure (38). Our present results demonstrate that inhibition of p38 and JNK blunted sustained Ado-induced EC apoptosis and mitochondria defects. Next, we assessed if sustained exposure to elevated Ado activates mitochondria-mediated apoptosis. We found that sustained exposure to elevated Ado for 24 and 48 h activated caspase-3 (Fig. 5A), also activated caspase-9 (Fig. 5A), and also activated caspase-8 (Fig. 5A), a marker for activation of mitochondria-mediated intrinsic pathway of apoptosis. In addition, the levels of proapoptotic protein, Bax, was reduced, and the apoptotic proteins, Bax and Bad, were elevated (Fig. 5B). The ratio of Bax to Bcl-2 was significantly increased (Fig. 5C). These results suggest that sustained Ado exposure causes lung EC apoptosis via alteration of Bcl-2 family proteins and subsequent mitochondria-mediated activation of caspase-9.

**Inhibition of xanthine oxidase had no effect on sustained Ado-induced caspase-3 activation.** Ado is metabolized to hypoxanthine by intracellular ADA, ultimately leading to generation of H2O2 if xanthine oxidase (XO) is activated. However, ADA inhibitor, DCF, has been shown to completely inhibit intracellular ADA activity and block conversion of exogenous [14C]Ado to hypoxanthine (30). Accordingly, we found that the XO inhibitor, allopurinol, did not alter sustained Ado-induced apoptosis.
images from three independent experiments are shown. stripped and reprobed for actin to control for protein loading. Representative caspase-3 cleavage (Fig. 6). Thus our data confirm that intracellular ADA activity is inhibited by DCF in our model and that the XO-mediated pathway is unlikely to play a role in sustained Ado-induced endothelial apoptosis.

DISCUSSION

Ado has been shown to be protective against apoptosis and tissue injury via activation of AR-mediated signaling (14, 18, 54, 55, 57). Our laboratory has previously shown that sustained Ado exposure causes pulmonary EC barrier dysfunction via NT-mediated intracellular Ado uptake (38). In this study, we further demonstrate that sustained Ado exposure causes pulmonary EC apoptosis via NT-dependent, p38- and JNK-mediated activation of mitochondrial apoptotic pathway. We also show that CS exposure increases lung tissue Ado levels in mice, an effect associated with lung EC apoptosis and early emphysema.

Lung endothelial apoptosis has been implicated in the pathogenesis of a variety of lung diseases, such as ALI, emphysema, and I/R lung injury (40). Plasma Ado is significantly elevated in patients with sepsis-induced ALI (43, 60) and tissue ischemia (59). Increased plasma Ado is associated with increased mortality in septic patients (43, 60). Ado levels are also increased in BAL fluid of human smokers (20). However, it is unknown whether lung tissue Ado levels are increased in smokers or patients with emphysema. ADA activity was decreased in plasma of rats exposed to CS for 4 wk (62) and in lungs of patients with chronic obstructive pulmonary disease (70), suggesting that lung tissue Ado levels may be elevated by CS exposure. In this study, we demonstrate that prolonged exposure (3 wk) to CS elevated lung tissue Ado levels in AKR mice, an effect associated with development of early emphysema and lung EC apoptosis. Extracellular ATP contributes to CS-induced lung inflammation and emphysema via purinergic receptors of inflammatory cells (16, 41, 47). ATP is a precursor of Ado. Whether elevated lung Ado plays a role in CS-induced lung endothelial apoptosis and emphysema remains to be determined. Nevertheless, our results demonstrate correlations between elevated lung tissue Ado and CS-induced emphysema and lung EC apoptosis. Studies from ADA-deficient mice have revealed that chronic elevation of Ado is associated with increased alveolar cell apoptosis and emphysema-like changes (8, 15, 69). Thus sustained elevation of lung Ado may contribute to CS-induced lung endothelial apoptosis and the development of emphysema.

Extracellular Ado can act via cell surface G protein-coupled AR and/or uptake into cells by NTs. Sustained Ado exposure in ADA-deficient mice enhanced alveolar cell apoptosis, an effect that was worsened in A2BR/ADA double-deficient mice (69), suggesting that prolonged Ado exposure-induced alveolar cell apoptosis is not due to activation of A2BR. Consistent with this in vivo effect, we show that inhibition of any one of the ARs exacerbated sustained Ado-induced apoptosis of cultured lung EC. These results suggest that AR-mediated signaling limits sustained Ado-induced EC apoptosis. Similar to other G-protein coupled receptors, prolonged exposure to Ado leads to desensitization and internalization of AR (24). Therefore, we speculate that sustained elevated Ado is taken up into cells by NTs, leading to enhancement of intracellular Ado levels that, in turn, triggers AR-independent effects. EC predominantly express equilibrative NT 1 (ENT1) and ENT2 with ENT1 expressed at twice the level of ENT2 (3). ENT1 has a 2.8-fold higher affinity for Ado than ENT2 (68). Concentrative NTs have very low affinity for Ado and limited expression in EC (35). We found that DPM and NBTI, highly specific inhibitors for ENT1 (~1,000-fold less effective on ENT2) (5, 65), completely prevented sustained Ado exposure-induced EC apoptosis and loss of mitochondrial membrane potential, suggesting that ENT1 may play a major role in Ado transport into EC and may mediate sustained Ado-induced EC apoptosis. Specific molecular approaches are needed to confirm the role of ENT1 in future studies. Nevertheless, our results demonstrate that sustained Ado exposure causes lung EC apoptosis via NT-mediated intracellular uptake, rather than by AR-mediated signaling.

Our laboratory has previously demonstrated that elevated extracellular Ado causes lung endothelial apoptosis via increased ratio of intracellular S-adenosylhomocysteine (SAH) to S-adenosyl-l-methionine (SAM) due to inhibition of SAH hydrolase (56). Consistently, a recent study also showed that exogenous Ado causes endothelial apoptosis and oxidative stress.

![Fig. 6. Effects of xanthine oxidase (XO) inhibitor on sustained adenosine exposure-induced EC apoptosis. Bovine PAEC were treated with V or 50 μM A plus 50 μM D in the absence or presence of allopurinol (AP; 100 μM) for 24 h, and apoptosis was assessed by procaspase-3 cleavage. Immunoblots were stripped and reprobed for actin to control for protein loading. Representative images from three independent experiments are shown.](http://ajplung.physiology.org/)

![Fig. 7. Proposed model: Sustained exposure to elevated adenosine (Ado) causes endothelial apoptosis via NT-dependent activation of p38 and JNK and subsequent MT defects and ultimately activation of MT pathway of apoptosis. PM, plasma membrane. The solid lines indicate defined pathways, and the dashed lines indicate speculative pathways.](http://ajplung.physiology.org/)
stress via accumulation of intracellular SAH (58). The downstream signaling following accumulation of SAH mediating Ado-induced apoptosis remains unknown. SAM is synthesized exclusively in the cytosol (53) and then transported into mitochondria (2). Mitochondrial SAM is essential for maintenance of mitochondrial function due to its critical role as a precursor to glutathione. SAM has been shown to elevate glutathione levels in hepatocytes and prevent alcohol-induced mitochondrial dysfunction via attenuation of oxidative stress in rats (4, 12, 29). Our laboratory has shown that sustained exposure to elevated Ado increases reactive oxygen species (ROS) levels and activates p38 and JNK in lung EC mitochondria (38). Both p38 and JNK are redox-sensitive proteins activated upon oxidative stress (45). ROS-mediated p38 activation has been implicated in extracellular ATP-induced macrophage apoptosis (49) and $H_2O_2$-induced endothelial apoptosis (42). Activation of p38 has also been implicated in homocysteine-induced apoptosis of endothelial progenitor cells (6) and of cardiomyocytes (67). In this study, we show that inhibition of p38 and JNK prevents sustained Ado-induced mitochondrial defects and endothelial apoptosis. Our data suggest that sustained A do exposure may increase intracellular SAH levels, thus reducing the availability of SAM to mitochondria, which leads to mitochondrial oxidative stress and subsequent activation of p38 and JNK in mitochondria, and ultimately resulting in endothelial apoptosis.

The antioxidant, NAC, and NADPH oxidase inhibitor, apocynin, were not able to rescue sustained Ado-induced apoptosis. We speculate that this may be due to the inability of these reagents to inhibit oxidative stress in mitochondria. We also show that the XO inhibitor, allopurinol, did not alter sustained Ado-induced caspase-3 cleavage, indicating that the XO-mediated pathway unlikely plays a role in sustained Ado-induced ROS generation and apoptosis.

Active p38 and JNK inhibit Bcl-2, thus causing apoptosis via direct phosphorylation of Bcl-2 (19, 23). Activation of p38 also mediates ischemia-induced apoptosis of isolated cardiomyocytes via increasing Bax mitochondrial translocation and accumulation (11). We show that sustained Ado exposure decreased Bcl-2, increased Bax and Bad, and significantly increased the ratio of Bax to Bcl-2. These effects were associated with activation of p38 and JNK, loss of mitochondrial membrane potential, caspase-9 activation, and apoptosis. Thus our data indicate that sustained exposure to Ado causes lung EC apoptosis via p38- and JNK-dependent activation of mitochondrial-mediated apoptotic pathway (Fig. 7).

In summary, we show that CS exposure increased lung tissue A do levels in mice, an effect associated with early emphysema and lung EC apoptosis. Using an in vitro model of sustained Ado exposure, we demonstrated that sustained Ado exposure caused lung EC apoptosis via NT-mediated intracellular Ado uptake, activation of p38 and JNK in mitochondria, subsequent mitochondria defects, and ultimately activation of the mitochondria-mediated apoptotic pathway. Our data reconcile the paradoxical effects of Ado on apoptosis in that prolonged exposure causes apoptosis via NT-mediated intracellular Ado uptake, whereas acute exposure is protective via activation of AR. Our data also suggest that sustained elevation of Ado in the lungs may contribute to CS-induced lung EC apoptosis and lung injury.

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

AUTHOR CONTRIBUTIONS

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