Cellular localization of adenine receptors in the rat kidney and their functional significance in the inner medullary collecting duct

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Kishore BK, Zhang Y, Gevorgyan H, Kohan DE, Schiedel AC, Müller CE, Peti-Peterdi J. Cellular localization of adenine receptors in the rat kidney and their functional significance in the inner medullary collecting duct. Am J Physiol Renal Physiol 305: F1298–F1305, 2013. First published August 28, 2013; doi:10.1152/ajprenal.00254.2013.—The G<sub>i</sub>-coupled adenine receptor (AdeR) binds adenine with high affinity and potentially reduces cellular cAMP levels. Since cAMP is an important second messenger in the renal transport of water and solutes, we localized AdeR in the rat kidney. Real-time RT-PCR showed higher relative expression of AdeR mRNA in the cortex and outer medulla compared with the inner medulla. Immunoblots using a peptide-derived and affinity-purified rabbit polyclonal antibody specific for an 18-amino acid COOH-terminal sequence of rat AdeR, which we generated, detected two bands between 30 and 40 kDa (molecular mass of native protein: 37 kDa) in the cortex, outer medulla, and inner medulla. These bands were ablated by preadsorption of the antibody with the immunizing peptide. Immunofluorescence labeling showed expression of AdeR protein in all regions of the kidney. Immunoperoxidase revealed strong labeling of AdeR protein in the cortical vasculature, including the glomerular arterioles, and less intense labeling in the cells of the collecting duct system. Confocal immunofluorescence imaging colocalized AdeR with aquaporin-2 protein to the apical plasma membrane in the collecting duct. Functionally, adenine (10 μM) significantly decreased (P < 0.01) 1-deamino-8-D-arginine vasopressin (10 nM)-induced cAMP production in ex vivo preparations of inner medullary collecting ducts, which was reversed by PSB-08162 (20 μM, P < 0.01), a selective antagonist of AdeR. Thus, we demonstrated the expression of AdeR in the renal vasculature and collecting ducts and its functional relevance. This study may open a new avenue for the exploration of autocrine/paracrine regulation of renal vascular and tubular functions by the nucleobase adenine in health and disease.

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Adenine receptor.

tor antagonist suramin could change the inhibitory effect of adenine on isoproterenol-induced cAMP formation in human 1321N1 astrocytoma cells stably expressing mouse AdeRs (33a). Using site-directed mutagenesis, the same group (20) showed that two amino acid residues (Asn194 and Leu201) are crucial for the activation of rat AdeRs (20). Finally, they cloned and characterized a new adenine receptor in the mouse, designated Ade1R, as opposed to the previously described mouse AdeR, Ade2R (31).

Important functions of the kidney, such as the transport of water and Na⁺ across the tubular cells, are mediated through cAMP or Ca²⁺ signaling. Apart from circulating hormones, such as vasopressin and aldosterone, these signaling pathways are known to be modulated by autocrine/paracrine agents, such as extracellular ATP, endothelins, and prostaglandins (PGE₂) through their respective G protein-coupled receptors. Our laboratories have long-standing interest in the autocrine/paracrine regulation of the function of the distal nephron (3, 9, 17, 19, 36–38). Hence, we studied the expression and cellular localization of AdeR in the rat kidney. Using gene-specific primers, we determined the relative mRNA expression of AdeR in the rat kidney. We also designed and generated a peptide-derived rabbit polyclonal antibody specific for AdeR and immunolocalized the receptor protein in the rat kidney. In addition, we documented the functionality of AdeR in ex vivo preparations of medullary collecting ducts (CDs), using adenine as an agonist and PSB-08162 as a selective antagonist of AdeR.

Thus, this study may open a new avenue for exploration of the autocrine/paracrine regulation of medullary collecting ducts (CDs), using adenine as an agonist and PSB-08162 as a selective antagonist of AdeR.

**METHODS**

**Experimental animals.** Rats (Sprague-Dawley) used in this study were obtained from the Charles River Breeding Lab (Wilmington, MA). Animals were maintained in specific pathogen-free condition and had free access to drinking water and rodent chow. The use of these animals was approved by the Institutional Animal Care and Use Committees of the Veterans Affairs Salt Lake City Health Care System (Salt Lake City, UT) and the University of the Southern California (Los Angeles, CA). Animals were euthanized, and kidney and brain tissue samples were collected and processed for mRNA or protein detection or immunoperoxidase/immunofluorescence labeling by methods previously established in our laboratories (11, 12, 36, 38–40).

Detection of AdeR mRNA. The approach of real-time RT-PCR established in our laboratory was used to determine the relative AdeR mRNA expression in different regions of the kidney (36, 38–40). Briefly, kidney tissue was dissected into six regions, namely, the superficial and deep cortex, outer and inner stripes of the outer medulla, and the base and tip of the inner medulla. Total RNA was extracted from each region, and traces of genomic DNA were removed. cDNA was synthesized by SuperScript Reverse Transcriptase II (Invitrogen, Carlsbad, CA). Real-time PCR amplifications were carried out on cDNA samples in an Applied Biosystems 7500 Real-Time PCR System (Foster City, CA) using AmpliTaq Gold and SYBR green for detection. Expression levels of AdeR were computed relative to the expression of β-actin mRNA in the samples. The sequences of primer pairs used are shown in Table 1. The specificity of PCR amplifications was confirmed by sequencing the PCR products in the DNA Core Facility of the University of Utah and then blasting nucleotide sequences obtained in the National Center for Biotechnology Information database.

**Rat AdeR sequence used to synthesize the immunizing peptide compared with the corresponding peptide sequences of the two types of mouse AdeRs**

<table>
<thead>
<tr>
<th>Position of Amino Acid Residues</th>
<th>Amino Acid Residues</th>
<th>Position of Amino Acid Residues</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat AdeR</td>
<td>293</td>
<td>G S F R H Q* K* H Q T L K M V L* Q R* A</td>
</tr>
<tr>
<td>Mouse Ade1R</td>
<td>293</td>
<td>G S F R H R L* Q* H Q T L K M V I* Q S* A</td>
</tr>
<tr>
<td>Mouse Ade2R</td>
<td>293</td>
<td>G S F R H R L* K* H Q T L K M V L* Q S* A</td>
</tr>
</tbody>
</table>

*Amino acid residues that do not match.*
F1300 AdeR EXPRESSION IN THE RAT KIDNEY

Synthesis and characterization of PSB-08162. PSB-08162 [3-methylamino-N-(9H-purin-6-yl) propanamide dihydrochloride], a selective antagonist of AdeR, was synthesized, purified, and characterized in the laboratory of C. Müller at the University of Bonn (Bonn, Germany). The compound was validated for its selective antagonism toward rat AdeR stably transfected in 1321N1 astrocytoma cells. Details of the synthesis and characterization of PSB-08162, which constitute a doctoral thesis work, will be published elsewhere.

Detection of AdeR protein. The immunoblot approach established in our laboratory was used for the detection of AdeR protein in the brain and kidney (36, 39, 40). Briefly, the rat brain and kidney cortex, outer medullary, and inner medullary tissue samples were homogenized in a buffer containing protease inhibitors, and protein contents of the homogenates were determined and then solubilized in Laemmli sample buffer. Proteins in the solubilized samples were size fractionated by electrophoresis on 12% polyacrylamide gels under denaturing conditions. Proteins in the gel were electrotransferred to nitrocellulose membranes. After being blocked with 2% goat serum (Jackson Immunoresearch Labs, West Grove, PA) in blocking buffer, membranes were incubated with affinity-purified AdeR antibody (1:500 dilution) or the antibody preadsorbed with a 10-fold molar excess of the immunizing peptide overnight at 4°C. After washoff of the primary antibody, membranes were incubated with horseradish-peroxidase-conjugated secondary antibody to goat IgG (Dako North America, Carpinteria, CA). Sites of antigen-antibody reaction were detected by a chemiluminescence reaction and captured on X-ray film.

Localization of AdeR protein by immunoperoxidase labeling. Localization of AdeR protein by immunoperoxidase labeling was carried out as previously described with a few modifications (14, 15). Briefly, rat kidney samples were fixed in 10% neutral buffered formalin for 2 days and then embedded in paraffin. After deparaffinization, thin sections (5 μm) were either stained with hematoxylin-eosin for conventional histology or processed for immunoperoxidase labeling. For immunoperoxidase labeling, antigen retrieval was achieved by incubating sections with 1% SDS in PBS (pH 7.4) for 5 min at room temperature. Sections were then washed with PBS and treated for the removal of endogenous peroxidase activity. Afterward, sections were blocked for 1 h at room temperature in blocking buffer (5% BSA and 0.5% Tween 20 in PBS). Sections were then incubated at 4°C overnight with affinity-purified rabbit polyclonal antibody against AdeR in blocking buffer at a dilution of 1:50. To establish the specificity of the AdeR antibody, control kidney sections were incubated in parallel with preimmune IgG (4 μg/ml). After washoff of the primary antibody, sections were incubated with ABC reagent (Vector Laboratories, Burlingame, CA) for 30 min at room temperature followed by color development with 3,3′-stain Elite ABC Kit, Vector Laboratories, Burlingame, CA) for 30 min. To confirm antibody specificity, control kidney sections were incubated in parallel with preimmune IgG (4 μg/ml). After washoff of the primary antibody, sections were incubated with ABC reagent (Vector Laboratories, Burlingame, CA) for 30 min at room temperature followed by color development with 3,3′-diaminobenzidine reagent (Vector Laboratories). After a wash, sections were counterstained with hematoxylin, dehydrated, and mounted with Permount under coverslips. Processed tissue sections were examined under a Reichert microscope, and digital pictures were taken with a Nikon 995 Coolpix camera.

Localization of AdeR protein by confocal immunofluorescence. Localization of AdeR protein by confocal immunofluorescence was performed as previously described (11, 12). Kidneys were fixed in situ by perfusion of 4% paraformaldehyde, and tissue blocks containing all organ regions were then placed in the same fixative overnight at 4°C. Tissue blocks were embedded in paraffin, and 4-μm sections were cut, deparaffinized in toluene, and rehydrated through xylene and graded ethanol. To retrieve antigens, slides were heated for 2 × 10 min in a microwave with medium heat in PBS and allowed to cool for 40 min. Sections were then fixed with 4% paraformaldehyde for 10 min and permeabilized for 10 min with 0.1% Triton X-100 in PBS. Goat serum (1:20 dilution, Jackson Immunoresearch Labs) in PBS was applied to sections for 1 h to block nonspecific binding. Sections were then probed with AdeR rabbit polyclonal antibody at a dilution of 1:100 overnight followed by an incubation with secondary Alexa fluor 594-conjugated goat anti-rabbit antibody (Invitrogen) at 1:500 dilution for 1 h. Some sections were double labeled first by an incubation with an aquaporin-2 (AQP2) mouse monoclonal antibody (1:500) followed by an incubation for 1 h with goat anti-mouse secondary Alexa fluor 488-conjugated antibody at a dilution of 1:500. After a final wash step, all sections were mounted with Vectashield mounting media containing the nuclear stain 4’,6-diamidino-2-phenylindole (Vector Laboratories) and examined with a Leica TCS SP5 confocal microscope.

Effect of adenine on 1-deaminoo-8-arginine vasopressin-stimulated cAMP generation in the rat inner medullary collecting duct. The effects of adenine on 1-deaminoo-8-arginine vasopressin (dDAVP) stimulation of cAMP generation, and the specific involvement of AdeR in it, were determined in freshly prepared fractions enriched in rat inner medullary collecting ducts (IMCD) by the methods previously established in our laboratories (36, 39, 40). Briefly, inner medullas were minced and digested with collagenase and hyaluronidase to obtain small fagments of the IMCD. IMCD fragments in the digest were isolated from the rest of the non-IMCD elements by low-speed centrifugation and washes, aliquoted into microtubes, and then processed for incubation with test substances. All incubations were performed in the presence of 0.5 mM IBMX to inhibit phosphodiesterases. Aliquots of the fractions enriched in IMCD, after being warmed to 37°C, were incubated for 10 min with or without the addition of 10 μM adenine (Sigma Chemical, St. Louis, MO). This was followed by the addition of 10 nM dDAVP (Sigma) to some aliquots, and the incubation was continued for another 20 min at 37°C. PSB-08162 was first dissolved in DMSO and diluted in incubation buffer. PSB-08162 was added to respective aliquots of the IMCD fractions at a final concentration of 20 μM just before fractions were warmed to 37°C. The reaction was stopped by adding chilled 0.1 N HCl. IMCD fragments were pelleted by centrifugation, and cAMP levels in the pellets were quantified by a CoomassiePlus Protein Assay Reagent Kit (Pierce Biotechnology). Measured cAMP levels in the samples were normalized to the protein contents of the respective pellets.

RESULTS

Relative expression of AdeR mRNA in the rat kidney. Using gene-specific primers for rat AdeR, we determined the relative mRNA expression of AdeR in different regions of the rat kidney. Real-time RT-PCR showed expression of AdeR mRNA in all regions of the kidney. However, expression levels were relatively higher in the deep cortical region and outer medulla and lowest in the inner medullary tip. The superficial cortex and base of the inner medulla showed modestly higher levels of AdeR mRNA (Fig. 1).

Characterization of AdeR antibody. To study the expression and intrarenal localization of AdeR protein, affinity-purified rabbit polyclonal antibody specific for an 18-amino acid COOH-terminal sequence of rat AdeR was generated. Immunoblot analysis detected two bands between ~30 and 40 kDa (molecular mass of native protein: 37 kDa) in samples of the rat kidney cortex, outer medulla, and inner medulla and in control brain tissue, which were ablated by preadsorption of the antibody with the immunizing peptide (Fig. 2A). To further confirm antibody specificity, immunoperoxidase labeling of rat kidney sections was performed with or without the AdeR antibody. Intense labeling was found in blood vessels in the rat renal cortex with the affinity-purified rabbit polyclonal AdeR antibody, but there was a lack of labeling in parallel-run kidney sections when the preimmune IgG fraction was used (Fig. 2B).
Expression and immunolocalization of AdeR protein in the rat kidney. By immunofluorescence labeling, we found high levels of expression of AdeR protein in all major regions of the kidney (Fig. 3). The labeling appeared to be localized in tubular as well as vascular structures. In most kidney regions except the tip of the papilla, AdeR protein expression (Fig. 3) was consistent with the RT-PCR data shown in Fig. 1. The predominant expression of AdeR protein in the vasculature and collecting duct system was confirmed by immunoperoxidase labeling. As shown in Fig. 4, A and B, the muscular layers of small- and medium-sized arteries were intensely labeled. In addition, there was clear labeling of glomerular arterioles (Fig. 4B). However, glomerular cells were not la-

Fig. 1. Relative mRNA expression of adenine (Ado) receptor (AdeR) in different regions of the rat kidney. Kidneys from four rats were dissected into different regions, namely, the superficial cortex (S-Ctx), deep cortex (D-Ctx), outer stripe of the outer medulla (O-Om), inner stripe of the outer medulla (I-Om), base of the inner medulla (B-Im), and tip of the inner medulla (T-Im). Tissue samples from each animal were processed separately for RNA extraction followed by real-time RT-PCR for rat (r)AdeR and β-actin, as described in METHODS. The mRNA expression of AdeR was computed relative to the mRNA expression of β-actin in the respective samples. Mean values are plotted as percentages, taking the highest value as 100%. Bars show means ± SE for four rats.

Fig. 2. Specificity of the AdeR antibody (Ab). A: Western blot analysis. Two identical blots were prepared by applying 25 μg protein of solubilized whole tissue homogenates of the brain (cerebral cortex), renal cortex (CTX), inner medulla (IM), or outer medulla (OM) of the rat. Blots were probed with affinity-purified rabbit polyclonal AdeR Ab (left) or Ab preadsorbed with a molar excess of the immunizing peptide (right). B: immunoperoxidase labeling on paraffin-embedded tissue sections. Blood vessels in the rat renal cortex were clearly labeled with affinity-purified rabbit polyclonal AdeR Ab (left), and there was a clear absence of peroxidase labeling of blood vessels when the preimmune IgG fraction was used in a parallel kidney section.

Fig. 3. Overview of the expression of AdeR protein in the rat kidney using immunofluorescence. A: cortical regions. B: OM regions. C: IM regions. AdeR protein was detected in all major regions of the rat kidney and appeared to be localized in tubular as well as vascular structures. Bars = 100 μm.
beled for AdeR. Weak labeling was found in the proximal tubules. Interestingly, weak but clear labeling was seen over clusters of peritubular interstitial cells in the cortex (Fig. 4E).

In the medulla, there was weak and diffuse labeling of the thick ascending limbs (not shown here). On the other hand, AdeR immunolabeling was clear and continuous along the tubular epithelium in the medullary collecting duct (Fig. 4, C and D). The labeling was predominant at the apical aspects, although diffuse intracellular labeling was seen in the collecting duct cells. Medullary interstitial cells were not labeled for AdeR protein (Fig. 4F).

To further explore the nature of AdeR-expressing cells in the medullary collecting duct and to study whether AdeR is expressed along with AQP2 protein in cells, we performed double-labeling immunofluorescence of AdeR and AQP2. AdeR immunofluorescence was seen all over medullary collecting duct cells (Fig. 5A, red color), similar to AQP2 immunofluorescence (Fig. 5B, green color). Overlay of the AdeR and AQP2 immunofluorescence profiles clearly showed colocalization of these two proteins in medullary collecting duct cells and revealed apical cell membrane colocalization of AdeR with AQP2 (Fig. 5C, yellow color).

Effect of adenine on dDAVP-stimulated cAMP production in the rat IMCD. Since double-immunofluorescence labeling revealed the colocalization of AdeR and AQP2 protein along the apical membrane of medullary collecting duct cells, we further
explored the functional relevance of renal AdeR expression in IMCD cells. For this, we tested the effect of adenine on dDAVP-induced production of cAMP in ex vivo preparations of the IMCD as well as its blockade by a selective antagonist of AdeR. As shown in Fig. 6, the addition of dDAVP (10 nM) to rat IMCD preparations caused a 2.4-fold increase in cAMP levels (vehicle control: 0.78 ± 0.10 pmol/µg protein vs. dDAVP: 1.91 ± 0.51 pmol/µg protein, P < 0.001), which was significantly reduced by coincubation of dDAVP with 10 µM adenine (0.61 ± 0.21 pmol/µg protein). Thus, these data indicate that adenine is a potent inhibitor of dDAVP-induced cAMP production in IMCD cells. To further explore whether the effect of adenine on dDAVP-induced cAMP production in the IMCD is mediated via AdeR or not, we used PSB-08162, a selective antagonist of AdeR, which was synthesized and characterized by C. Müller and associates. To ensure robust inhibition, in our incubations we used a twofold molar excess of PSB-08162 (20 µM) relative to the adenine concentration (10 µM). As shown in Fig. 6, PSB-08162 significantly (P < 0.01) reversed the inhibitory effect of adenine on dDAVP-induced cAMP production in the IMCD. PSB-08162 alone did not have any effect on basal or dDAVP-induced cAMP levels in the IMCD. These data indicate that the inhibitory effect of adenine is mediated via AdeR and not independent of AdeR, thus establishing the functionality of AdeR in the IMCD.

DISCUSSION

This study describes the expression, cellular localization, and functional significance of AdeRs in the rat kidney. Using gene-specific primers and a peptide-derived polyclonal antibody, we showed that AdeR mRNA and protein are highly expressed in the rat kidney. The predominant AdeR immunolabeling was found in the cortical vasculature and in cells of the collecting duct system. Both medium and small arteries as well as glomerular arterioles were labeled in the cortex. In the collecting duct, AdeR was colocalized to the apical plasma membrane along with AQP2 protein. In parallel, we showed that adenine significantly reduced dDAVP-induced cAMP production in ex vivo preparation of the rat IMCD, and this effect was specifically mediated via AdeR. These findings suggest that adenine may have potential autocrine/paracrine regulatory roles in the renal vasculature and collecting duct. Thus, this study opens a new avenue for exploration of the autocrine/paracrine regulation of renal tubular and vascular functions by the nucleobase adenine. We discuss the significance of our findings in relation to renal physiology or pathophysiology below.

In mammalian systems, adenine in the extracellular milieu is not derived from the degradation of adenine-containing nucleotides. Most cells can synthesize and recycle adenine, which makes adenine a potential autocrine or paracrine agent. Our immunolocalization experiments showed strong labeling for AdeR in the collecting duct system, colocalized with AQP2 protein. This indicates that AdeR is expressed in principal cells of the collecting duct, although its expression in other cells,
such as intercalated cells, was seen in immunofluorescence. Since the cAMP signaling pathway is crucial for the function of collecting ducts, our data suggest that extracellular adenine may play potentially significant autocrine/paracrine regulatory roles in water and/or acid/base transport in the collecting duct. This notion is supported by our findings showing that adenine significantly inhibited dDAVP-stimulated cAMP generation in ex vivo preparations of the IMCD, and this action was specifically mediated via AdeR.

In healthy humans, the plasma concentration of adenine is ~70 nM. However, in patients with chronic renal failure (CRF), plasma adenine levels are elevated to higher than 1 μM (29). The high plasma adenine levels in patients with CRF are directly correlated with the severity of kidney failure, as assessed by serum creatinine concentrations. Interestingly, plasma adenine concentrations in patients with CRF also directly correlated with erythrocyte ATP concentrations and the adenine incorporation rate into the erythrocyte adenine nucleotide pool. Based on these findings, it has been suggested that elevated plasma adenine concentrations, in addition to the accelerated incorporation into the erythrocyte adenine nucleotide pool, may play an important role in the raised ATP concentration in uremic patients (29). A significant reduction in both plasma adenine and erythrocyte ATP was observed immediately after hemodialysis, but 2 days later, the high predialysis plasma adenine and erythrocyte ATP concentrations were restored (29). However, after successful renal transplantation, erythrocyte ATP and plasma adenine concentrations reached control values (29). Interestingly, the ability of blood adenine to increase the erythrocyte ATP concentration is the basis for the inclusion of significant amounts of adenine in CPD-1 blood preservative for the long-term storage of human blood for transfusion (24, 28).

These observations in patients with CRF underscore the importance of adenine and AdeR and the significance of our study. Although the physiological effects of circulating adenine in the kidneys of healthy humans is not known, it is conceivable that elevated blood concentrations of adenine in conditions such as CRF may have significant effects mediated through the recently characterized AdeR depending on the sites of its intrarenal expression. Recently, it has been reported that AdeRs are expressed in primary rat hepatic stellate cells (HSC) as well as T-6 cells, an immortalized rat HSC cell line, and these cells undergo functional changes in response to adenine (34). The adenine-induced functional changes in HSCs are actin reorganization, inhibition of the inositol 1,4,5-trisphosphate-mediated increase in cytosolic Ca\(^{2+}\), inhibition of chemotaxis, and upregulation of α-smooth muscle actin and collagenase type 1 (34). Thus, this report suggests that adenine has a potential signaling role in cellular differentiation. Future studies should investigate whether adenine has a similar signaling role in cellular differentiation in the kidney, especially in conditions such as CRF.

Although in our study on the rat kidney we could not find the expression of AdeR protein in proximal tubular cells, it is possible that AdeR might be expressed in proximal tubular cells of other species. This notion is supported by a report (35) showing that adenine inhibited Na\(^{+}\)-ATPase in isolated basolateral membranes of proximal tubular cells of the pig kidney via G\(_i\)-coupled inhibition of the cAMP signaling pathway. Although bulk absorption of Na\(^{+}\) in the proximal tubule is mainly driven by ouabain-sensitive Na\(^{+}\)-K\(^{+}\)-ATPase or the Na\(^{+}\) pump, however, ouabain-insensitive and furosemide-inhibitable Na\(^{+}\)-ATPase, often called the second Na\(^{+}\) pump, may be involved in fine tuning the Na\(^{+}\) reabsorption in the proximal tubule (for a review, see Ref. 26). Experimental evidence suggests that Na\(^{+}\)-ATPase is a target for angiotensins and that it is increased in the kidneys of spontaneously hypertensive rats, without alterations in the expression of Na\(^{+}\)-K\(^{+}\)-ATPase (6, 26). In view of these reports, future studies should investigate the potential role of AdeR on Na\(^{+}\) reabsorption in the kidney and intestines of different species.

In conclusion, we localized the AdeR in the rat kidney and showed its functional role in IMCD cells. Based on its cellular localization, the AdeR may play important functional roles in renal hemodynamics and salt and water transport. Thus, this work opens a new avenue to study the role of adenine in renal physiology and pathophysiology based on the precise localization of AdeR.

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

AUTHOR CONTRIBUTIONS

REFERENCES


