A regulated NH$_2$-terminal Sgk1 variant with enhanced function is expressed in the collecting duct

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1Department of Internal Medicine, University of Iowa College of Medicine, Iowa City, Iowa; 2Graduate Program in Molecular Biology, University of Iowa College of Medicine, Iowa City, Iowa; and 3Veterans Affairs Medical Center, Iowa City, Iowa

Submitted 3 April 2012; accepted in final form 30 September 2012

Raikwar NS, Liu KZ, Thomas CP. A regulated NH$_2$-terminal Sgk1 variant with enhanced function is expressed in the collecting duct. Am J Physiol Renal Physiol 303: F1527–F1533, 2012. First published October 3, 2012; doi:10.1152/ajprenal.00191.2012.—Sgk1 is a relatively unstable kinase that regulates epithelial Na$^+$ transport in the distal nephron of the kidney. We identified a 5$'$ variant alternate transcript of human Sgk1 (Sgk1_v3) that is expressed in the connecting tubule and collecting duct, is regulated by aldosterone and insulin, and is predicted to encode an NH$_2$-terminal variant Sgk1 isoform, Sgk1_i3. Sgk1_i3 contains a polybasic motif, KKR, in its NH$_2$ terminus that regulates ubiquitination and stability of the expressed protein in HEK293 cells. In Fisher rat thyroid, and mpkCCDc14 cells, Sgk1_i3 had a significantly greater effect on Na$^+$ transport compared with Sgk1 and its stimulatory effect was dependent on the kinase domain. Sgk1_i3 increased the abundance of cleaved epithelial Na$^+$ channel (ENaC) subunits at the cell surface, which was inhibited by coexpression of Nedd4-2. Together, the data demonstrate that a renally expressed Sgk1 isoform, Sgk1_i3, shows improved stability, is regulated by insulin and aldosterone, and stimulates ENaC activity when heterologously expressed in collecting duct cells.

aldosterone; Na$^+$ transport; epithelial Na$^+$ channel

NA$^+$ reabsorption in the principal cells of the late distal convoluted tubule (DCT), connecting tubules (CNT), and collecting ducts (CD) of the kidney occurs via the amiloride-sensitive epithelial Na$^+$ channel (ENaC). This channel, a heteromultimeric protein complex, is expressed at the apical membrane of epithelial cells and consists of three structurally related subunits: α-, β-, and γ-ENaC (10, 22).

A number of studies established that serum and glucocorticoid-regulated kinase 1 (Sgk1), a serine/threonine kinase closely related to the PKB/Akt family, is an important early regulator of ENaC-mediated Na$^+$ transport in the DCT, CNT, and CD of the kidney (18, 26). Mice with germline inactivation of Sgk1 demonstrate Na$^+$ wasting while on a low-salt diet; findings that have recently been confirmed in an inducible renal-specific knockout mouse (11, 12, 28). Sgk1 is rapidly induced by serum, growth factors, and corticosteroid hormones and is activated by phosphorylation which is stimulated by a signaling cascade that includes phosphoinositide 3-kinase (PI 3-kinase), the PIP-3-dependent kinase, PDK1, and the kinases WNK1 and mTORC2 (4, 16, 17). In turn, activated Sgk1 increases ENaC activity through multiple pathways including an increase in transcription and translation of α-ENaC, an increase in surface retention of the channel complex, and an increase in open probability of the channel (10, 16, 29).

Sgk1 is an unstable protein that is rapidly degraded following ubiquitin modification of multiple lysines within the NH$_2$ terminus (2, 7). We and others previously reported that a highly conserved alternate transcript of Sgk1, termed Sgk1_v2, encodes NH$_2$-terminal variant Sgk1 isoform, Sgk1_i2 (Sgk1.1), that is more stable and increases the activity of a number of cation channels of the ENaC/dengenerin family (1, 20, 27). Although expressed in the CD, this isoform is not regulated by corticosteroids and its relevance in aldosterone-regulated distal nephron Na$^+$ transport may be limited. We now identify another functional Sgk1 isoform, Sgk1_i3, that is expressed in the CD, stimulates ENaC activity, and is regulated by insulin and aldosterone.

MATERIALS AND METHODS

Materials. Aldosterone, amiloride, dexamethasone, insulin, selenium, sorbitol, transferrin, triiodothyronine, and N-acetyl-Leu-Leu-Norleucinal (ALLN) were purchased from Sigma (St. Louis, MO) and cycloheximide was purchased from EMD Chemicals (San Diego, CA). All cell culture media were obtained from Invitrogen Life Technologies (Gaithersburg, MA). Anti-FLAG M2 antibody was obtained from Sigma, anti-HA antibody from US Biological (Swampscott, MA), anti-α-tubulin antibody and horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG were from Santa Cruz Biotechnology (Santa Cruz, CA), and HRP-conjugated goat anti-rabbit IgG from Cell Signaling Technology (Danvers, MA).

Cell culture. Human embryonic kidney cell line HEK293 was cultured in high-glucose DMEM containing 10% FBS and 1% penicillin-streptomycin. MpkCCD14 cells were cultured in defined media containing 2% FBS, antibiotics, and other hormones, as previously described (3). Fisher rat thyroid (FRT) cells were cultured in F-12 Coon’s media (Harlan, Indianapolis, IN) with 5% fetal calf serum (Sigma), 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C, as previously described (23). M-1 cells were maintained in DMEM/F-12 containing 10% FBS and 1% penicillin-streptomycin.

Plasmids. ENaC subunits with COOH-terminal tags α-ENaC-FLAG, β-ENaC-V5, γ-ENaC-Myc, and Sgk1-FLAG and Nedd4-2 have been described previously (14, 20, 21). To generate human Sgk1_i3 with a COOH-terminal FLAG epitope (Sgk1_i3-FLAG), the 5′ end of Sgk1_V3 was PCR amplified using forward primer hSgk1V3_For containing an EcoRV site and reverse primer hSgk1V3_Rev containing a PacI site: ccagATCTGACGTCAAGGACGGCAC

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and Sgk1_i3 contain conserved polybasic clustered residues. The instability motif in Sgk1, GMVAIL is shown.

**Transgenic transfections, communoprecipitation, cell surface biotinylation, and Western blotting.** FRT cell transfection was done as previously described (20), whereas mpkCCDc14 cells were transfected in suspension by Nucleofection (Lonna, Walkersville, MD), using SF 4D-Nucleofector X solution according to the manufacturer’s directions. HEK293 cells were grown in cell culture dishes until subconfluent and then transiently transfected with Lipofectamine 2000 (Invitrogen) as per manufacturer’s recommendation. Forty-eight hours following transfection, cells were lysed directly for Western blotting or for immunoprecipitation followed by Western blotting. In some experiments, cells were treated with 10 μM ALLN in serum-free media overnight before lysis. In other experiments, 20 μg/ml of cycloheximide were added to transfected cells in serum-free media for various times. Cell lysis, communoprecipitation, cell surface protein biotinylation, and Western blotting were performed as published (20).

**Total RNA isolation and cDNA preparation.** Total RNA from mouse kidney cortex, medulla, and papilla was isolated with TRIzol (Life Technologies, Grand Island, NY) and then further purified with Absolutely RNA miniprep kit (Agilent Technologies). RNA from cDNA synthesis was also prepared with the Absolutely RNA miniprep kit. RNA from microdissected distal nephron segments was extracted with Absolutely RNA Nanoprep kit (Agilent Technologies). RNAs were subject to reverse transcription with AffinityScript quantitative PCR cDNA synthesis kit (Agilent Technologies) under the following conditions: 25°C for 5 min for primer annealing, 42°C for 45 min for cDNA synthesis, and 95°C for 5 min for termination. Other cDNAs were purchased as a human tissue panel (CLONTECH, Mountain View, CA).

**Real-time PCR.** Primers used in real-time PCR for human and mouse Sgk1, Sgk1_V2 have been published (20). Mouse sgk1_V3 from cells and tissues was amplified using a forward primer msgk1_V3_F: 5′-AGGCTCAGAAAAGGAGCGAGTCC and reverse primer msgk1_V3_R: 5′-TTAGCGTTCATAAGCTCCGGCTCC. For human Sgk1_V3, we used forward primer hSgk1_V3_F: 5′-AAAGGAGC-GAGTCCITCTCG and reverse primer hSgk1_V3_R: 5′-GGGTGTG-GCATTCATAAGCTCCGGCTCC. Quantitative real-time PCR was performed as described before (20).

**Electrophysiology.** Short-circuit measurements (Isc) in transfected FRT cells were performed as previously described (23). Nucleofected mpkCCDc14 cells were seeded on collagen-coated transwell filters (12-mm-diameter Millicell-PCF, Millipore) and short-circuit currents were measured as described previously (21).

**Statistics.** Data are provided as arithmetic means ± SE. All data were tested for significance with a Student’s t-test or one-way ANOVA where applicable, using SigmaStat (SPSS, Chicago, IL). In all cases, P values <0.05 were considered statistically significant.

**RESULTS**

We and others previously reported the primary structure and possible function of Sgk1_v2, an alternate transcript of Sgk1 that arises in exon 1a~120 kb upstream of the transcription start site of human Sgk1 and splice to exons 1b, 1c, and 2 through 12 (Fig. 1A) (20). Computational analysis of gene and transcript databases reveals the presence of at least two additional 5′ human variants, which we term Sgk1_v3 and Sgk1_v4, where exons 1d and 1e, respectively, splice to exon 2. Each of the three alternate transcripts encodes NH2-terminal variant proteins Sgk1_i2, Sgk1_i3, and Sgk1_i4 (Fig. 1B). Sgk1_v3 like Sgk1_v2 is widely conserved from platypus to human while sequence corresponding to exon 1e (Sgk1_v4) have only been reported in genome databases of primates (NCBI RefSeq, data not shown). We performed a pairwise comparison of human, rhesus, and mouse sequences at the 5′ end of the Sgk1 locus between exons 1e and 2 using the alignment tool zPicture (http://zpicture.dcode.org/ ). The homology between human and rhesus is extremely high at each of these exons and their intervening sequences, but the homology drops off substantially between human and mouse at exon 1e (Fig. 1C), suggesting that Sgk1_v4 may not have an ortholog.

![Fig. 1. NH2-terminal serum and glucocorticoid-regulated kinase 1 (Sgk1) variants. A: intron-exon organization of human SGK1. Reference sequence Sgk1 arises from exons 1 through 12. The most 5′ exon identified is ∼140 kb upstream of exon 1. B: schematic of 5′ human sgk1 variants. Exons 1a, 1b, and 1c splice to exon 2 through 12 to create Sgk1_v2, while exon 1d and 1e splice to exon 2 and beyond to create Sgk1_v3 and Sgk1_v4, respectively. C: pairwise comparison of the human, rhesus, and mouse genomic sequence at the Sgk1 locus using the alignment tool zpicture (zpicture.dcode.org) shows conserved regions with a similarity between 50 and 100%. D: unique NH2 termini of human Sgk1 isoforms. Conserved basic residues are shown bolded and underlined. Both Sgk1_i2 and Sgk1_i3 contain conserved polybasic clustered residues. The instability motif in Sgk1, GMVAIL is shown.](http://ajprenal.physiology.org/)

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in rodents. A BLAST search of the mouse and rat genome using the human exon 1e sequence did not show any similarity and attempts to amplify exon 1e from mouse genomic DNA were unsuccessful (data not shown). We therefore focused our studies on Sgk1_v3. A comparison of the NH2 termini of the Sgk1 isoforms demonstrates that both Sgk1_i2 and Sgk1_i3 contain clustered polybasic residues and do not share the motif GMVAIL that contributes to the instability of Sgk1 (Fig. 1D) (5).

We examined Sgk1_v3 expression in a human tissue mRNA panel and demonstrate that this transcript, like Sgk1, is widely expressed (Fig. 2). In mouse cortical CD (CCD) and in the mouse CD cell lines, mouse distal nephron segments including the DCT, CNT, and the cortical CD (CCD) and in the mouse CD cell lines, M-1 and mpkCCDc14 (Fig. 2, B, C, and D). We tested the effect of insulin and aldosterone on Sgk1 mRNA expression in mpkCCDc14 cells, as these hormones are known to regulate Na+/H+ transport in the CNT and CCD. The results demonstrate that insulin and aldosterone increase the expression of Sgk1 and Sgk1_v3 but not Sgk1_v2, suggesting that the increase in Sgk1_v3 expression may contribute to the increase in Na+ transport (Fig. 2E).

Since Sgk1_i3 has a predicted NH2 terminus different from Sgk1, we hypothesized that this isoform may be more stable compared with Sgk1. The steady-state abundance of Sgk1_i3 was much greater than Sgk1 when equivalent amounts were expressed from the same plasmid vector in HEK293 cells. We then assessed the impact of proteasomal inhibition with ALLN and do not share the motif GMVAIL that contributes to the instability of Sgk1 (Fig. 1D) (5).

We expressed Sgk1_i3 with ENaC subunits in FRT epithelia and demonstrate a significant increase in Isc compared with ENaC alone (Fig. 4A). We mutated lysine at position 141 (K141N) to abolish the kinase domain and demonstrate that this construct is no longer able to stimulate Na+ transport. We then tested Sgk1_i3 constructs in mpkCCDc14 cells and directly compared these with Sgk1. The results, as in FRT cells, demonstrate that Sgk1_i3, but not Sgk1_i3K141N, stimulates Isc (Fig. 4B). Sgk1 and Sgk1_i3 were comparable in their ability to stimulate Isc. We compared the expression level of the Sgk constructs in these cells. There appeared to be considerably more Sgk1_i3 expression compared with Sgk1 (Fig. 4C), likely related to its enhanced stability as shown by the studies in HEK293 cells. The inability of Sgk1_i3K141N to stimulate Isc is not related to lower levels of expression, indicating that the kinase domain of Sgk1_i3 is required for the stimulatory effect on Na+ transport.

The NH2 terminus of Sgk1_i3 contains a polybasic motif KKR that may be a target for ubiquitination during the normal turnover of Sgk1_i3. We mutated the KKR sequence to NNG (KKRmut) and when expressed noted that the steady-state levels of Sgk1_i3 KKRmut were much higher than that of wild-type Sgk1_i3 (Fig. 5A). We measured the decay of Sgk1_i3 KKRmut compared with Sgk1_i3 and demonstrate the increased abundance of Sgk1_i3 KKRmut is related to its
longer half-life (Fig. 5B). We compared the effect of both forms on \( \text{Na}^+ \) transport in mpkCCDc14 cells and confirm that Sgk1_i3 KKRmut has a greater stimulatory effect on \( I_{sc} \) compared with Sgk1_i3 (Fig. 5C). As in HEK293 cells, Sgk1_i3-KKRmut expression was greater than Sgk1_i3 in mpkCCDc14 cells (data not shown). Finally, we examined the polyubiquitination of Sgk1_i3 and show that wild-type Sgk1_i3 is ubiquitinated to a much greater extent compared with Sgk1_i3 KKRmut (Fig. 5, D and E). These results strongly indicate that ubiquitination of Sgk1_i3 is at least partly at the KKR motif and disruption of this motif further increases the half-life of Sgk1_i3 thus regulating its abundance and its functional effect on \( \text{Na}^+ \) transport.

Sgk1 is reported to increase ENaC activity in several ways including the phosphorylation and inactivation of Nedd4–2, a ubiquitin ligase that promotes endocytosis,

\[
\begin{align*}
\text{ENaC} + \text{Sgk1_i3} & \quad \text{ENaC} + \text{Sgk1_i3 K141N} \\
\text{Veh} & \quad \text{Sgk1} \\
\end{align*}
\]

Fig. 4. Effect of Sgk1 on \( \text{Na}^+ \) transport. A: Fisher rat thyroid (FRT) epithelia were transfected with \( \alpha, \beta, \gamma \)-epithelial \( \text{Na}^+ \) channel (ENaC) alone or with either Sgk1_i3 or Sgk1_i3K141N; \( n = 3 \) exp of 4 filters each. \(* P < 0.05 \) for Sgk1_i3 compared with control. **\( P < 0.001 \) Sgk1_i3 compared with Sgk1_i3K141N. B: mpkCCDc14 epithelia were subjected to nucleofection with empty plasmid, Sgk1, Sgk1_i3, or Sgk1_i3K141N; \( n = 3 \). **\( P < 0.001 \) compared with vehicle and compared with Sgk1_i3K141N. Sgk1_i3 stimulates amiloride-sensitive short-circuit measurement (\( I_{sc} \)) in FRT and mpkCCDc14. C: immunoblot of mpkCCDc14 cells subjected to nucleofection with various plasmids and then grown on filters, resolved by SDS-PAGE, and probed with FLAG and tubulin antibodies. Abundant Sgk1_i3 and Sgk1_i3K141N (top band) is seen compared with Sgk1 (arrow).

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ubiquitination, and degradation of ENaC subunits (8, 24). Since Nedd4–2 induces degradation of proteolytically cleaved ENaC subunits (15), we wondered whether Sgk1_i3 could stimulate the proteolytic cleavage of ENaC, thus increasing the activity of surface resident channels. We used a surface biotinylation assay to assess the abundance and cleavage of total and surface expressed ENaC in HEK293 cells. We demonstrate cleavage of surface-expressed ENaC but not total cellular ENaC. Expression of an increasing amount of Sgk1_i3 increased the abundance of cleaved ENaC subunits at the cell surface (Fig. 6, A and B). We tested the effect of Nedd4–2 on Sgk1_i3-mediated ENaC cleavage. We find that when Nedd4–2 is overexpressed, there is a reduction in the Sgk1_i3 dose-dependent cleaved ENaC subunits evident at the cell surface. These studies indicate that Nedd4–2 can interact with and inhibit the effect of Sgk1_i3 (Fig. 6, C and D).

DISCUSSION

New information from large-scale genome and transcriptome sequencing has led to the identification of a number of alternate transcripts for any given gene. In the case of Sgk1, a highly conserved serine threonine kinase first described in 1993, the diversity of the proteome arises in part from multiple transcription start sites that lead to alternate transcripts that encode NH2-terminal variants. One of these transcripts, termed Sgk1_v2 or Sgk1.1, is more selectively expressed (1).

Sgk1 contains an instability motif in the NH2 terminus that leads to its rapid degradation by ubiquitination resulting in a protein with a very short half-life (7). Given the role of the NH2-terminal variant, and degradation of ENaC subunits (8, 24). Since Nedd4–2 induces degradation of proteolytically cleaved ENaC subunits (15), we wondered whether Sgk1_i3 could stimulate the proteolytic cleavage of ENaC, thus increasing the activity of surface resident channels. We used a surface biotinylation assay to assess the abundance and cleavage of total and surface expressed α-ENaC subunits in HEK293 cells. We demonstrate cleavage of surface-expressed ENaC but not total cellular ENaC. Expression of an increasing amount of Sgk1_i3 increased the abundance of cleaved ENaC subunits at the cell surface (Fig. 6, A and B). We tested the effect of Nedd4–2 on Sgk1_i3-mediated ENaC cleavage. We find that when Nedd4–2 is overexpressed, there is a reduction in the Sgk1_i3 dose-dependent cleaved ENaC subunits evident at the cell surface. These studies indicate that Nedd4–2 can interact with and inhibit the effect of Sgk1_i3 (Fig. 6, C and D).

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terminals in regulating the abundance of Sgk1, we were interested in studying these NH2-terminal variants of Sgk1. Sgk1_v2 encodes an NH2-terminal protein isoform, Sgk1_i2, which was reported to be more stable and preferentially membrane localized but translocates to the cytosol upon PLC stimulation (1, 20).

Sgk1_i3, the encoded product of Sgk1_v3, is widely conserved and like Sgk1 is expressed in multiple tissues and in particular in the cortex, medulla, and papilla of the kidney and in the DCT, CNT, and CCD, distal nephron segments where ENaC is expressed. Importantly, we confirmed that insulin and aldosterone stimulate Sgk1_v3 expression in the CD cell line, mpkCCD14.

When expressed in HEK293 cells we confirmed that Sgk1_i3 was more stable than Sgk1 and that this correlated with lower levels of ubiquitination. Interestingly, ALLN, a proteasomal inhibitor, also increased the abundance of Sgk1_i3, although this was not statistically significant, suggesting that lysine residues elsewhere in the protein or in the variant NH2 terminus may also be targets for ubiquitination. We confirmed that the increased steady-state abundance of Sgk1_i3 was secondary to a longer protein half-life and that this correlated with reduced ubiquitination of Sgk1_i3 compared with Sgk1. We found that mutation of a KKR sequence in the NH2 terminus further enhanced the stability of Sgk1_i3 which was attributable to a reduction in ubiquitination and presumably its proteasomal degradation. The increased stability of this mutated form of Sgk1_i3 enhanced its ability to increase Na+ transport. The simplest explanation is that the lysine residues in the KKR sequence are the targets for ubiquitin modification.

We tested the effect of Sgk1_i3 in FRT epithelia and in mpkCCD14 cells and demonstrated that Sgk1_i3 significantly stimulated Na+ transport. In mpkCCD14 cells, Sgk1_i3 and Sgk1 had a similar effect on Na+ transport even though Sgk1_i3 was expressed at a higher level compared with Sgk1. Sgk1 may stimulate ENaC-mediated Na+ transport via multiple pathways, each involving phosphorylation of intermediate substrates such as Nedd4-2, WNK4, and Af9 (9, 13, 29). Furthermore, Sgk1 forms a multiprotein complex with ENaC, the ENaC regulatory complex (ERC), which includes adapter molecules, scaffold proteins, and signaling proteins (25). Sgk1 and Sgk1_i3 differ in their NH2 termini which may regulate its interaction with various NH2 termini account for the relative efficiency of these isoforms to increase Ic. We predicted that the function of Sgk1_i3 would be dependent on the kinase domain that is present in the common COOH terminus. As expected, mutation of the kinase domain abolished the effect of Sgk1_i3 on Na+ transport in both FRT and mpkCCD14 cells. Furthermore, Sgk1_i3 increases surface expression of cleaved ENaC channels and this was inhibited by overexpression of Nedd4-2, indicating the Sgk1_i3, like Sgk1, interacted with Nedd4-2 to regulate surface expression and activity of ENaC channels. In previous work, we and others showed that Nedd4-2 overexpression reduces surface expression by increasing the endocytosis and degradation of cleaved ENaC channels (15, 21). The reduction in surface-expressed cleaved channels seen here with Nedd4-2 could be secondary to enhanced endocytosis and removal of the Sgk1_i3 stimulated cleaved subunits or to an inhibition of Sgk1_i3 leading to a reduction in subunit cleavage (30).

Studies in Sgk1-null mice demonstrate that Sgk1 is an important regulator of urinary Na+ and K+ excretion and plays a role in fetal programming of blood pressure and the extracellular volume expansion seen with the insulin-sensitizing agent pioglitazone (6, 16, 28). We demonstrate that Sgk1_v3 is widely expressed, including in the distal nephron, similar to Sgk1. Furthermore, Sgk1_v3 mRNA is regulated by aldosterone and insulin, two physiologically important mediators of Na+ transport in the CD. Further studies will need to be done to determine whether individual Sgk1 variants are differen-
tially expressed or regulated in various nephron segments. A study of the regulation of Sgk1 isoforms in vivo has been hampered by the lack of good isomorf-specific antibodies. The effect of differing stimuli such as aldosterone and IGF-1 stimulation of individual Sgk1 variants may contribute to the integration of different signaling pathways that impact Na⁺ transport in the distal nephron.

ACKNOWLEDGMENTS

The authors thank C. Zurzulo for the FRT cell line, A. Vandewalle for mpkCCDc14 cell line, John B. Stokes and Rita Sigmund for microdissected tubular segment RNA, and D. Bohmann for the HA-tagged ubiquitin vector. The authors also acknowledge the University of Iowa DNA Core facility and Vector Core facility for services provided.

The nucleotide sequence reported in this paper will appear in DDBJ, EMBL, Genbank, and GSDB Nucleotide Sequence Databases with accession number JQ836654. The suggested names for the alternate Sgk1 transcript (Sgk1_v3) and protein isoform (Sgk1_v3) in this manuscript are based on guidelines from the HUGO Gene Nomenclature Committee (HGNC) at http://www.genenames.org.

GRANTS

This work was supported in part by United States Public Health Service Grant HL71664, DK090053, and by a Veterans Affairs Merit Review Award.

DISCLOSURES

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

AUTHOR CONTRIBUTIONS


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