Glucose stimulates neurotensin secretion from the rat small intestine by mechanisms involving SGLT1 and GLUT2, leading to cell depolarization and calcium influx

**Rune Ehrenreich Kuhre,* Louise Ellegaard Bechmann,** Nicolai Jacob Wewer Albrechtsen, Bolette Hartmann, and Jens Juul Holst

**NNF Center for Basic Metabolic Research and Department of Biomedical Sciences, Panum Institute, Faculty of Health and Medical Sciences, University of Copenhagen, Copenhagen, Denmark**

Submitted 8 January 2015; accepted in final form 20 April 2015

Glucose stimulates neurotensin secretion from the rat small intestine by mechanisms involving SGLT1 and GLUT2, leading to cell depolarization and calcium influx. Am J Physiol Endocrinol Metab 308:E1123–E1130, 2015. First published April 21, 2015; doi:10.1152/ajpendo.00012.2015.—Neurotensin (NT) is a neuropeptide produced in the central nervous system and in the gut epithelium by the enteroendocrine N cell. NT may play a role in satiety regulation, as centrally administered NT is released from the intestine in response to nutrient stimulation and may have potential in obesity treatment. Glucose ingestion stimulates NT secretion in healthy young humans, but the mechanisms involved are not well understood. Here, we show that rats express NT in the gut and that glucose gavage stimulates secretion similarly to oral glucose in humans. Therefore, we conducted experiments on isolated perfused rat small intestine with a view to characterize the cellular pathways of secretion. Luminal glucose (20% wt/vol) stimulated secretion but vascular glucose (5, 10, or 15 mmol/L) was without effect. The underlying mechanisms depend on membrane depolarization and calcium influx, since the voltage-gated calcium channel inhibitor nifedipine and the K<sub>ATP</sub> channel opener diazoxide, which causes hyperpolarization, eliminated the response. Luminal inhibition of the sodium-glucose cotransporter 1 (SGLT1) (by phloretin) eliminated glucose-stimulated release as well as secretion stimulated by luminal methyl-α-D-glucopyranoside (20% wt/vol), a metabolically inactive SGLT1 substrate, suggesting that glucose stimulates secretion by initial uptake by this transporter. However, secretion was also sensitive to GLUT2 inhibition (by phlorizin) and blockade of oxidative phosphorylation (2,4-dinitrophenol). Direct K<sub>ATP</sub> channel closure by sulfonylureas stimulated secretion. Therefore, glucose stimulates NT secretion by uptake through SGLT1 and GLUT2, both causing depolarization either as a consequence of sodium-coupled uptake (SGLT1) or by closure of K<sub>ATP</sub> channels (GLUT2 and SGLT1) secondary to the ATP-generating metabolism of glucose.

**NEUROTENSIN (NT)** is a 13-amino acid peptide neuromodulator, first isolated from bovine intestine and brain tissue (3, 14). It is stored in secretory vesicles in enteroendocrine N cells in the intestine and in neuronal vesicles in the CNS. NT is released from the intestine in response to nutrient stimulation and may play a role in satiety regulation, as centrally administered NT decreases food intake in rodents (19, 20). A few reports have shown that glucose stimulates NT secretion in healthy young humans (18) and from isolated perfused rat small intestine (4), but the underlying mechanisms of secretion are not well understood. Studies of secretion of the gut hormone glucagon-like peptide-1 (GLP-1) have indicated that glucose-stimulated secretion of this hormone involves electrogenic sodium uptake through sodium-glucose transporter 1 (SGLT1) as well as closure of ATP-sensitive potassium (K<sub>ATP</sub>) channels (6, 17, 21, 24). Recent findings indicate that some enteroendocrine cells have substantial similarities in respect to expression of glucose transporters and molecular glucose sensors (5, 8). Therefore, we hypothesized that the mechanisms of glucose-stimulated NT could have similarities with the mechanisms of glucose-stimulated GLP-1 secretion. As an initial approach, we investigated whether the rat expresses NT in the gut and whether glucose intake (by gavage) stimulates NT secretion from rats. As this was the case, and because the glucose gavage-stimulated secretion profile was similar to that observed in humans (18), we decided to use the rat as a model animal for further investigations. However, as plasma NT concentrations are low and because intact, biologically active NT<sub>1-13</sub> is extremely rapidly metabolized by plasma endopeptidases [t<sub>1/2</sub> < 2 min in humans (11)], generating COOH-terminally truncated forms (15), characterization of the mechanisms underlying its secretion cannot easily be carried out in vivo. We therefore investigated the mechanisms underlying glucose-stimulated NT secretion using isolated perfused rat small intestine, since there the N cell has its normal localization in the gut epithelium with maintained cell polarity (enabling discrimination between apical and basolateral activated mechanisms), contact with nerves, vascular supply, and the normal paracrine relationship to the neighboring cells. At the same time, the model allows application of activators/inhibitors of proteins that may play a role for NT secretion but would be hazardous or lethal in vivo (e.g., nifedipine).

**MATERIALS AND METHODS**

**Rat tissue and protein extraction.** Tissue was collected in a previous study (16). In brief, tissue was harvested at 8–11 AM from nonfasted rats (weight 274 ± 12 g) anesthetized by subcutaneous injection with Hypnorm-Midazolam (0.2 ml/100 g, conc. 5 mg/ml). Samples (~1.5 cm/piece) were collected from duodenum, proximal jejunum, distal ileum, caecum, proximal colon, and distal colon, thoroughly rinsed in cold PBS, snap-frozen on dry ice, and stored at −80°C until protein extraction. For protein extraction, tissue was homogenized in 1% (vol/vol) trifluoroacetic acid (TFA; cat. no. TS-28904, Thermo Fisher Scientific) with a 5-mm steel bead and a bead mill (30 Hz for 2 × 2 min; TissueLyzer, Qiagen Instruments, Hombrechtikon, Switzerland). Samples were left to stand for 1 h at room temperature and thereafter cleared by centrifugation (3,300 g, 10 min).
MO) (pH 0.6 mmol/l Thiomersal (cat. no. T-5125; Sigma Chemical, St. Louis, MO), 20 mmol/l EDTA, and 0.6 mmol/l Thiomersal (cat. no. T-5125; Sigma Chemical, St. Louis, MO) (pH = 8.5). Anatomic definitions of the respective gut sections are described elsewhere (16).

Glucose gavage in rats. Studies were carried out with permission from the Danish Animal Experiments Inspectorate (2013-15-2934-00833) and the local ethics committee (EMED, P-14-213) according to previously described procedures with minor modifications (18). In brief, male Wistar rats were obtained from Taconic (Ejby, Denmark) and allowed at least 1 wk acclimatization before study. Each day of the week preceding the study, rats were handled to accustom them to the procedures, which included restraint and gavage feeding, here with drinking water. Experiments were carried out on “semifasted” rats just before their nocturnal feeding period (1700). Rats were divided into weight-matched groups (saline, 313 ± 1.5 g; glucose, 314 ± 4 g; P < 0.05, n = 6). At time 0 min, rats were given a gavage bolus of either glucose (2 g/1,000 g, 50% wt/vol) diluted in milli-Q water or a matched volume of milli-Q water (negative control). Blood (in total ~500 μl) was collected at time –10 (zero sample), 15, 30, and 60 min into prechilled EDTA-coated capillary tubes (cat. no. 200K3E, Microvette; Starstedt, Nümbrecht, Germany) by sublingual vein puncture (~100 μl/time point). Samples were instantly transferred onto ice and centrifuged (1,650 g, 4°C, 15 min) within 30 min after collection. Samples were stored at ~20°C until analysis. At the end of experiment, rats were anesthetized by subcutaneous (sc) injection of Hypnorm-Midazolam and killed by diaphragm perforation.

Intestinal rat perfusions. Samples were derived from a previous study (17) carried out with permission from the Danish Animal Experiments Inspectorate (2013-15-2934-00833) and the local ethics committee (EMED, P-12-099 and P-13-240) in accordance with the guidelines of Danish legislation governing animal experimentation (1987) and the National Institutes of Health (publication no. 85-23). Study design is described in greater detail elsewhere (17). In brief, male Wistar rats (~250 g) were obtained from Taconic (Ejby, Denmark) and housed two per cage with ad libitum access to standard chow and water, kept on a 12:12-h light-dark cycle. Nonfasted rats were anesthetized by sc injection with Hypnorm-Midazolam and placed on a 37°C heating table. The abdominal cavity was opened, and the entire large intestine and approximately two-thirds of small intestine were carefully removed, leaving the proximal part of the small intestine (~32 cm) in situ, therefore including the duodenal and jejunal sites of biopsy collection (Fig. 1A), but not the distal ileum. The length of the gut segment varied little (coefficient of variation 11.2%) between experiments. The gut was emptied of its content by flushing with isotonic saline (10 ml 0.9% NaCl, room temperature) after insertion of a plastic tube into the lumen. A steady flow (0.25 ml/min) was subsequently applied through the lumen. A catheter was inserted into the cranial/superior mesenteric artery and the gut was perfused (7.5 ml/min with perfusion buffer heated to 37°C using a Uniper UP-100 perfusion system; Hugo Sachs, Harvard Apparatus, March-Hugstetten, Germany). Venous effluent was collected via a metal catheter placed in the hepatic portal vein. Perfusion buffer consisted of Krebs-Ringer bicarbonate buffer supplemented with 0.1% BSA (albumin fraction V; Merck, cat. no. 1.12018.0500, Ballerup, Denmark), 5% dextan T-70 (to balance osmolality; Pharmacosmos, Holbaek, Denmark), 2% (vol/vol) amino acid mixture (Vamin, cat. no. 11338; Fresenius Kabí, Upsala, Sweden), 3.5 mmol/l glucose, 10 μM 3-isobutyl-1-methylxanthine (Sigma-Aldrich, cat. no. 5879) and 5 mmol/l pyruvate, fumarate, and glutamate, pH 7.4. To ensure adequate oxygen supply to the gut, the buffer was gassed with 95% O2-5% CO2, resulting in partial pressures (P: mmHg; means ± SE) of PO2 = 448 ± 54 and 494 ± 69, PCO2 = 30.4 ± 2.6 and 33.9 ± 3.9 at the start of each experiment, respectively (n = 7) in randomly chosen experiments. In the same line of experiments, P02 in venous effluent were reduced to ~60% (P < 0.0001), in samples collected at both the start and end of experiment, reflecting metabolic activity of the gut which did not alter over the course of the experiment.

The rat was euthanized by perforation of the diaphragm as soon as proper flow was apparent. After an equilibration period of 30 min, samples were collected each minute, immediately chilled on ice, and
transferred to −20°C within half an hour. The luminal stimuli were 20% (wt/vol) glucose, the nonmetabolizable SGLT1-specific sugar, methyl-α-α-glucopyranoside (α-MGP; 20% wt/vol), the nontransportable sugar mannitol (20% wt/vol), or the artificial sweeteners, sucrose (0.25% wt/vol) and ascorbic acid K 0.78% wt/vol), all applied at an initial rate of 2.5 ml/min for the first 2 min to replace the saline solution in the lumen, and then at 0.250 ml/min throughout the rest of the stimulation period. Glucose and α-MGP were added in presence or absence of the SGLT1 inhibitor phlorizin (10 mmol/l) or the GLUT2 inhibitor phloretin (1 mmol/l), applied to the lumen 10 min before the sugar stimulation as described above. Immediately after each stimulation, the lumen was flushed with a bolus of (0.9%) NaCl (2.5 ml/min) for 2 min, followed by infusion at a flow rate of 0.250 ml/min between stimulations. In other experiments, the gut was vascularly perfused with sulfonlureas (tolbutamid 500 μM or gliclazide 500 μM), the KATP channel opener diazoxide (500 μM), or the β-gated calcium channel blocker nifedipine (10 μM). Additional experiments assessed NT responses when glucose was administered intravascularly at physiological (5, 10, and 15 mmol/l) concentrations. Bombesin (BBS; 10 nM), a known enterocordore secretagogue, was included in all experiments as a positive control. All drugs were obtained from Sigma-Aldrich (Bredeney, Denmark). Drugs applied to the luminal side of the gut were diluted in isotonic NaCl (0.9% wt/vol) and stimulants for the vascular route in perfusion buffer. To aid solubility, diazoxide, gliclazide, lidocaine, nifedipine, phloretin, and tolbutamide were dissolved first in dimethyl sulfoxide (DMSO) and then further diluted in perfusion medium. DMSO content never exceeded a final concentration of 0.1%, which did not cause NT secretion in control experiments (n = 3; data not shown). In randomly chosen effluent samples, the health of the gut was assessed by measuring Po2, Pco2, lactate, and pH in perfusate collected from the arterial and venous sides of the preparation; later, histological examination of the tissue was carried out in randomly chosen specimens as described (17). In control studies, red blood cells (bovine, 20% vol/vol) were added to the perfusion buffer to test whether this would result in enhanced secretory function. The relative increase in NT secretion in response to 20% (wt/vol) luminal glucose did not differ between experiments with and without red blood cells [with: from baseline (0.94; 0.2 mmol/l to 30 min 8.05 ± 0.3 mmol/l, n = 6), whereas concentrations did not differ from control group, a minor increase in blood glucose was observed (0.94, 0.2 mmol/l), whereas concentrations did not differ (P < 0.05; Fig. 1A). Similar data were obtained for total NT (data not shown). Glucose gavage stimulates NT secretion from rats. To test whether the rat was a suitable model for studying the underlying mechanisms of glucose-stimulated NT secretion from humans, we gavage-stimulated Wistar rats with glucose or water (control). Blood glucose did not differ between groups at baseline (P = 0.94, n = 8). Glucose gavage elevated blood glucose at all tested time points (from baseline: 6.49 ± 0.2 mmol/l to 15 min 8.00 ± 0.3 mmol/l, 30 min 8.05 ± 0.32 mmol/l, 60 min 8.01 ± 0.3 mmol/l, P < 0.001 for all). In the control group, a minor increase in blood glucose was observed 30 min after gavage (from baseline: 6.5 ± 0.2 mmol/l to 30 min, 7.0 ± 0.2 mmol/l), whereas concentrations did not differ from baseline at the remaining time points (n = 8; Fig. 1B). Glucose gavage thus resulted in significantly higher blood glucose concentrations than water gavage at all tested points after baseline (P < 0.001). Plasma levels of total NT did not differ between groups at baseline (P = 0.77, n = 8). Glucose induced a rapid response at time point 15 min (from baseline: 7.3 ± 1.8 pmol/l to 13 ± 2.3 pmol/l, P < 0.05), but 30 and 60 min after stimulation concentrations were back to baseline (P > 0.05). Water gavage did not stimulate secretion at any time points (P > 0.05; Fig. 1C).
Glucose stimulates NT secretion by N cell depolarization, causing activation of V-gated calcium channels. Luminal glucose (20% wt/vol) stimulated NT secretion from the perfused rat intestine (from (3.6 ± 0.2 to 6.3 ± 0.6 pmol/l, n = 6, P > 0.001) but not in the presence of nifedipine (10 μM; from 3.0 ± 0.1 to 3.6 ± 0.1 pmol/l, n = 6, P > 0.05; Fig. 2, C and D). To assess whether this might be due membrane depolarization, we investigated the effects of hyperpolarizing the cell with the KATP-channel opener diazoxide. Luminal glucose again stimulated NT secretion (from 7.9 ± 1.1 to 17 ± 0.8 pmol/l).
PMONL/l), but this was completely lost by administration of dioxazide, and dioxazide even reduced the secretory rate during luminal glucose stimulation to below basal levels (from 5.0 ± 1.0 to 2.1 ± 0.2, n = 6, P < 0.001; Fig. 2, A and B). Control studies confirmed that repeated luminal glucose stimulation resulted in significant and similar responses (Fig. 2, A and B). In contrast, luminal stimulation with 20% (wt/vol) mannitol (osmolarity-control) did not stimulate secretion (4.2 ± 0.2 vs. 4.4 ± 0.1 pmol/l, n = 6, P = 0.42; Fig. 2, G and H).

Glucose stimulates NT secretion by SGLT1-mediated uptake. Luminal α-MGP stimulated NT secretion (from 6.7 ± 0.3 to 13 ± 1.0 pmol/l, n = 6, P < 0.0001) but not in the presence of luminally administered SGLT1 inhibitor (phloridzin, 10 mmol/l; Fig. 3, A and B). Similarly, secretory responses to luminal glucose (from 7.6 ± 0.3 to 14 ± 0.8 pmol/l, n = 6, P < 0.001) were abolished by coapplication of luminal phloridzin (Fig. 3, C and D).

NT secretion is stimulated by uptake through GLUT2 and K<sub>ATP</sub> channel closure. Luminal glucose stimulated NT secretion (from 7.6 ± 1.1 to 14 ± 0.8 pmol/l, n = 6, P < 0.001), but the response was also lost by luminal coadministration of the GLUT2 inhibitor phloretin (1 mmol/l; from 8.1 ± 0.2 to 8.3 ± 0.2 pmol/l, n = 6, P = 0.08; Fig. 4, A and B). K<sub>ATP</sub> channel closure by tolbutamide (500 μM) resulted in a rapid secretory response (from 5.3 ± 0.2 to 21 ± 7.7 pmol/l, n = 6, P = 0.11), whereas stimulation with gliclazide at same concentration resulted in a lower but still significant response (from 5.4 ± 0.2 to 9.5 ± 1.2 pmol/l, n = 6, P < 0.05; Fig. 4, C and D). Glucose-stimulated NT secretion (from 2.8 ± 0.4 to 8.7 ± 1.9 pmol/l) was eliminated by vascular administration of the oxidative phosphorylation blocker 2,4-dinitrophenol (from 2.7 ± 0.2 to 3.0 ± 0.2 pmol/l, n = 6, P > 0.05; Fig. 4, E and F).

Effect of vascular glucose and sweet-taste receptor activation. Vascular glucose did not stimulate NT secretion at 5 mmol/l concentration (P > 0.05), but at 10 and 15 mmol/l concentrations the secretory output increased minimally but significantly (10 mmol/l: from 9.1 ± 0.5 to 11 ± 0.6; 15 mmol/l: from 7.3 ± 0.4 to 8.3 ± 0.2, n = 6, P < 0.01 for both; Fig. 5, A and B). Luminal stimulation with the artificial sweeteners sucralose and acesulfame K, applied at a sweetness of 20 times that of glucose, had no effect on secretion (sucralose: from 3.2 ± 0.1 to 3.3 ± 0.1 pmol/l, acesulfame K: from 3.2 ± 0.9 to 3.2 ± 0.1 pmol/l, n = 6, P = 0.39 and 0.49, respectively; Fig. 5, C and D).

**DISCUSSION**

In our model, luminal glucose stimulated NT secretion from the perfused rat intestine, whereas vascular glucose had a small but significant effect when applied in upper physiological (10 mmol/l) and pathophysiological concentrations (15 mmol/l). Control experiments showed that the gut responded similarly to repeated luminal glucose stimulation and by mechanisms unrelated to osmolarity (as mannitol did not stimulate secretion). The glucose response appeared to depend on cell depolarization and opening of V-gated calcium channels, as diazoxide (K<sub>ATP</sub> channel opener; causing cell hyperpolarization) or nifedipine (V-gated calcium channel blocker) both abolished glucose-induced secretion. We next investigated the mechanisms driving the depolarization. Importantly, glucose failed to stimulate secretion when the SGLT1 transporter was inhibited (by phloridzin), consistent with a previous study (4). In further
support of the crucial role of SGLT1 for glucose-stimulated NT secretion, luminal stimulation with the SGLT1-specific, metabolically inactive sugar $\alpha$-MGP stimulated secretion, and this was also eliminated by phloridzin. However, luminal glucose-stimulated NT secretion was also blocked by inhibition of the electroneutral glucose transporter GLUT2 (by phloretin) and by blocking the oxidative phosphorylation (by 2,4-dinitrophenol). These findings suggest that glucose may also stimulate NT secretion by mechanisms similar to the pancreatic $\beta$-cell, where glucose is taken up by GLUT2 and metabolized intracellularly, generating a rise in [ATP], and secretion by $K_{ATP}$ channel closure and depolarization. In support of this hypothesis, direct closure of the $K_{ATP}$ channels by sulfonylureas stimulated NT secretion; tolbutamide caused a large but varying response (which therefore did not reach significance), whereas gliclazide caused a smaller but more consistent (significant) secretory response. Taken together, our data show that glucose stimulates NT secretion by cell depolarization and activation of V-gated calcium channels due to luminal uptake through SGLT1 and by GLUT2 activity, which work interdependently to stimulate secretion. Whereas the SGLT1-induced secretion can be explained by the sodium-coupled uptake (because $\alpha$-MGP stimulated secretion), a similar scenario cannot be established for GLUT2-stimulated NT secretion, as this transporter is electroneutral. Glucose transport via GLUT2, therefore, must result in activation of downstream effectors leading to membrane depolarization. One of these steps is probably $K_{ATP}$ channel closure, which caused NT secretion per se (sulfonylurea drugs). Supporting this, elimination of ATP generation by the oxidative phosphorylation uncoupler 2,4-dinitrophenol blocked the glucose response. Control studies showed that this was not due to general impairment of N cell function upon ATP depletion, because 2,4-dinitrophenol did not eliminate secretion by ATP-independent pathways (BBS). The classical model of glucose absorption states that glucose crosses the intestinal epithelium by active SGLT1-mediated uptake and exits basolaterally through GLUT2 (9, 28). However, during absorption of a glucose-containing meal, GLUT2...
may be recruited from the basolateral side of the cell (where it normally resides) to the apical side of the cell within minutes by mechanisms that depend on SGLT1 activity (6, 9, 12, 13). During assimilation of a meal, GLUT2 (low-affinity, high-capacity glucose transporter) may thereby assist SGLT1 (high-affinity, low-capacity) to increase the glucose absorption rate severalfold (13). On this basis, we propose that the underlying affinity, low-capacity) to increase the glucose absorption rate and GLUT2 in NT-producing cells by immunohistochemistry. It would be of interest to demonstrate expression of SGLT1 and GLUT2 in NT-producing cells by immunohistochemistry. However, SGLT1 has been detected on the luminal process of the GLP-1-producing L cell (6) and in expression analysis of purified murine L cells (23), and, like NT, GLP-1 secretion is sensitive to SGLT1 inhibition (6, 17, 21, 24). By analogy, therefore, we assume that these glucose transporters are also expressed by the NT cells, although a direct investigation of this is warranted. Furthermore, we cannot exclude that the administration of luminal phloretin may also have affected basolateral GLUT2 activity, which would be expected to impair the exit of glucose out of the cell and thereby perhaps prevent further glucose uptake.

For these studies, we chose to perfuse the proximal part, as this is the site where the majority of glucose absorption takes place. Consequently, the most NT-rich intestinal segment (distal ileum) was not part of the retained intestine. However, we (26) recently showed that the secretory NT outputs from the perfused upper and lower halves of the rat small intestine at baseline and in response to luminal peptones were similar, suggesting that the upper half is appropriate for secretion studies.

Taken together, our data indicate that glucose stimulates NT secretion via uptake into NT-secreting cells through both SGLT1 and GLUT2, leading to secretion by cellular depolarization and influx of calcium. Therefore, it appears that the secretory mechanisms responsible for glucose-stimulated NT secretion have striking similarity to the mechanisms of glucose-induced GLP-1 secretion. However, some studies indicate that the GLP-1 response to glucose results entirely from SGLT1 mediated transport, while others have shown that transport via GLUT2 + metabolism works to potentiate SGLT1 induced secretion (2, 6, 17, 21, 24). A concurrent view on these matters was recently published (7).

Recently, two independent groups have shown that FACS sorted enteroendocrine I or L cells from the mouse intestine [identified by their expression of a fluorescent marker controlled by the cholecystokinin (CCK) or proglucagon promoter, respectively] contain significant amounts of several other gut hormones than CCK and GLP-1, respectively, including NT (5, 8). The physiological implications of this and whether this translates into actual cosecretion of the costored hormones remains, however, unknown. In colocalization studies of endocrine cells in the rat gut mucosa, about 20% of the NT-producing cells costored GLP-1, but the majority did not (27).

Further investigations are needed to unravel the physiological role of NT in regulation of appetite and whether it should be explored further as a potential target for the treatment for obesity. Stimulating secretion of (endogenous) NT may turn out to be a beneficial therapeutic target, as agents capable of this might also stimulate secretion of other anorectic hormones from cells sharing similar secretory sensors (in particular CCK,
GLP-1, and oxyntomodulin); such agents would be expected to have pronounced effects on appetite because of potentiating interactions (25). Our findings suggest that NT secretion should always be considered in studies of appetite inhibition by carbohydrate-containing meals.

ACKNOWLEDGMENTS

This study was supported by a grant to J. J. Holst from the Novo Nordisk Centre for Basic Metabolic Research (Novo Nordisk Foundation, Denmark). The funding body had no influence on the design, conduction, interpretation or other aspects of this work.

DISCLOSURES

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

AUTHOR CONTRIBUTIONS


REFERENCES