Exendin-4 increases blood glucose levels acutely in rats by activation of the sympathetic nervous system

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The procedures described here were approved by the Institutional Animal Care and Use Committees at the University of Vigo and the University of Cincinnati. All experimental procedures were carried out in accordance with the European Union regulations regarding the

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EXENDIN-4-INDUCED ACUTE HYPERGLYCEMIA IN THE RAT

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protection of animals used for experimental purposes (Council Directive CEE 86/609) or in accordance with the National Institutes of Health (NIH) Guidelines for the Care and Use of Laboratory Animals.

Drugs and Peptides

GLP-1-(7–36)-NH2, hexamethonium (HXM), guanethidine (GU), D-glucose, and pentobarbital sodium were all provided by Sigma-Aldrich (Alcobendas, Spain). Ex-9 was obtained from Bachem (Bubendorf, Switzerland). Ex-4 was obtained from Sigma-Aldrich and Bachem. Vildagliptin was kindly provided by Dr. Bryan Burkey (Novartis Institutes for Biomedical Research). Sterile 0.9% NaCl solution was used as vehicle.

Experimental Protocols

Acute effects of peripheral Ex-4 on blood glucose. ACUTE EFFECTS OF EX-4 AND GLP-1 ON INTRAPERITONEAL GLUCOSE TOLERANCE IN ANESTHETIZED RATS. Male Sprague-Dawley (SD) (275–325 g) rats were anesthetized with pentobarbital sodium (50 mg/kg), and a silastic cannula was placed in the right jugular vein. Intravenous (iv) injections of saline, Ex-4 (5 µg/kg), or GLP-1-(7–36)-NH2 (20 µg/kg) were given immediately before an intraperitoneal (ip) bolus of glucose (1.5 g/kg D-glucose) and blood samples taken at fixed intervals for 120 min. A second group of rats was fasted for 48 h, anesthetized, and given iv saline or Ex-4 (0.5, 1, 5, or 20 µg/kg iv). The blood glucose response was also tested in 48-h-fasted rats given Ex-4 (5 µg/kg) or the GLP-1R antagonist exendin-(9–39) (100 µg/kg ip) together or separately. Finally, groups of rats were given low (15 µg/kg) or high (75 µg/kg) doses of GLP-1 with or without pretreatment with vildagliptin (12 µg/kg ip at time −15 min). We have found in previous studies that a similar pretreatment efficiently inhibits DPP IV activity (Aulinger BA and D’Alessio DA, unpublished data).

ACUTE EFFECTS OF EX-4 IN CONSCIOUS RATS. A permanent polyethylene catheter was placed in the right jugular vein under pentobarbital anesthesia (50 mg/kg) in SD rats 1 wk before challenge with Ex-4. On the day of the experiment, a heparin bolus (1,000 IU) was administered. Immediately, a blood sample was obtained and Ex-4 (5 µg/kg) was injected ip after an overnight fast. Blood glucose levels were monitored, and all animals, both chronic Ex-4 and saline treated, were injected with Ex-4 (15 µg ip). Blood glucose levels were monitored, with measurements taken at 0.5, 1, 3, and 6 h after the injection.

CHRONIC SUBCUTANEOUS INFUSION OF EX-4. Omotic minipumps (1007D; Durect) delivering Ex-4 or saline were implanted subcutaneously under isoflurane-induced anesthesia into the intrascapular space of male Long-Evans rats (300–350 g) after an overnight fast. Glucose levels were measured before anesthesia and 3 and 6 h after surgical implantation of the minipumps while food was withheld. Both groups had ad libitum access to food during the first dark phase to elucidate the effect of a continuous infusion of Ex-4 and saline on food intake. For the rest of the study, we gave the saline-treated rats the same amount of food that was eaten by the Ex-4-treated rats. After 6 days of treatment with Ex-4 or saline, rats were fasted overnight, and an IPGTT was done while the minipump infusions continued. Body composition was measured before surgery and after the IPGTT using NMR imaging (Whole Body Composition Analyzer; EchoMRI).

Metabolite and Hormone Measurements

Plasma glucose was measured using a commercial kit based on the glucose oxidase method (Biomerieux). Whole blood glucose samples were measured with standard glucose strips (Free Style; Abbott Laboratories). Insulin, corticosterone, C-peptide, and glucagon levels were measured with commercially available radioimmunoassay kits (DRG Systems, Marburg, Germany, for insulin and corticosterone; and Linco Research, St. Charles, MO, for C-peptide and glucagon) according to the manufacturer’s instructions. Metanephrine (MN) and normetanephrine (NMN) were determined using biochemical detection after reverse-phase isocratic HPLC following cationic exchange chromatography.

Statistical Analysis

The data are presented as means ± SE. Student’s t-test for independent samples was used for comparison between two groups. One-way ANOVA followed by Tukey’s post hoc test or two-way ANOVA followed by Bonferroni’s post hoc test was used for comparisons between multiple independent groups.
RESULTS

Acute Effects of Peripheral Ex-4 on Blood Glucose

Intravenous administration of GLP-1 or Ex-4 to anesthetized ad libitum-fed rats reduced the immediate glucose excursion after an ip bolus of glucose ($P < 0.05$ for the 0- to 15-min time points; Fig. 1A). This effect was accompanied by the expected increase of insulin secretion ($P < 0.05$ for the 5-min time point; Fig. 1B). In saline- and GLP-1-treated rats, glycemia was maximal after 15 min and returned to baseline by 120 min (Fig. 1A). In marked contrast, starting 30 min after the glucose bolus, blood glucose levels of the Ex-4 group increased continuously up to values of 280 mg/dl at 60 min and remained above 250 mg/dl for $\geq 2$ h (Fig. 1A). Despite the persistent hyperglycemia, plasma insulin levels in the rats given Ex-4 were comparable with the other groups.

The hyperglycemic activity of Ex-4 was reproduced in conditions in which the insulinotropic activity of the peptide is minimal, after 48 h of fasting in the absence of exogenous glucose (Fig. 1C). In this setting, Ex-4 caused a dose-dependent increase of plasma glucose 60 and 120 min after injection compared with saline-treated rats, with a threshold dose of 5 $\mu$g/kg to induce hyperglycemia (Fig. 1C). The hyperglycemic action of Ex-4 appeared to be mediated through the GLP-1R, since anesthetized rats pretreated with the GLP-1R antagonist Ex-9 before the administration of Ex-4 had glucose values similar to controls (Fig. 1D). In contrast to the acute hyperglycemia induced by Ex-4, GLP-1 did not have this effect, even when given at a very high dose and protected from rapid inactivation by coadministration of the DPP IV inhibitor vildagliptin (Fig. 1, E and F).

Peripheral administration of Ex-4 also induced a sustained increase in blood glucose levels in conscious rats at a dose that effectively reduces food intake. Ex-4 (5 $\mu$g/kg ip) significantly suppressed food intake 30 min after its administration in 24-h-fasted rats (Fig. 2A). The same dose of Ex-4 increased baseline glucose levels in freely moving overnight-fasted SD rats (Fig. 2B) and caused glucose intolerance following an ip bolus of glucose (Fig. 2C). These findings demonstrate that the acute effects of Ex-4 to cause hyperglycemia occur in conscious as well as anesthetized animals and are present in two commonly used strains of rats.

To determine whether there is a dose-dependent dissociation of Ex-4 action to improve glucose tolerance and induce hyperglycemia later in the GTT, a range of doses of Ex-4 were combined with an ip bolus of glucose (Fig. 2D). None of the doses (1, 5, and 20 $\mu$g/kg) of Ex-4 had a significant effect to improve ip glucose tolerance, and all caused hyperglycemia in the later phase of the test. This result indicates that, in the rat, acute hyperglycemia is the predominant response across the dose range tested.
Central Effects of Ex-4 on Blood Glucose

To determine the effects of CNS Ex-4 on blood glucose, Ex-4 was given icv to conscious rats in doses from 0.1 to 5 μg/rat. Intracerebroventricular Ex-4 increased fasting glucose levels in a dose-dependent fashion during 2 h of observation compared with saline-treated control rats (Fig. 3). These data indicate that both central and peripheral administration of Ex-4 can cause hyperglycemia.

The Hyperglycemic Effect of Ex-4 is Mediated Through the Sympathetic Nervous System

Because previous studies demonstrated effects of GLP-1 to activate the ANS, we hypothesized that hyperglycemia induced by Ex-4 is mediated though the ANS. To test this hypothesis, specific branches of the ANS were selectively blocked during peripheral and central Ex-4 administration. Fasted rats were pretreated with the preganglionic blocker HXM or saline, and Ex-4 or vehicle was given either ip (5 μg/kg; Fig. 4A) or icv (1 μg; Fig. 4B). HXM pretreatment alone had no effect on blood glucose levels in rats given saline ip or icv. Similarly to the previous experiments, Ex-4 caused an increase in blood glucose compared with control 1 h after its administration whether given ip or icv. This effect was completely blocked by HXM in both cases. These findings support a critical role for ANS signaling to mediate the acute effect of Ex-4 to raise blood glucose.

To determine the role of the sympathetic limb of the ANS in the hyperglycemic effect of acute Ex-4, fasted rats were pretreated with GU, which inhibits the release of norepinephrine from peripheral autonomic nerves, before ip administration of Ex-4 (20 μg/kg) or saline. Surgical vagotomy did not affect basal glucose levels compared with the sham-operated controls. Ex-4 significantly increased the blood glucose levels in control rats, but this effect was completely blocked by GU in rats with surgically disrupted vagus nerves. These findings suggest an important role of sympathetic nervous system (SNS) signaling to mediate the acute hyperglycemic effects of Ex-4 in rats.

To investigate the involvement of the parasympathetic limb of the ANS in the hyperglycemic actions of Ex-4, both branches of the vagus nerve were surgically disrupted at the cervical level in fasted, anesthetized Sprague-Dawley rats. Ex-4 caused an increase in blood glucose compared with control 1 h after its administration whether given ip or icv. This effect was completely blocked by HXM in both cases. These findings suggest a critical role for ANS signaling to mediate the acute effect of Ex-4 to raise blood glucose.

Fig. 2. Peripheral administration of Ex-4 increases blood glucose levels in conscious rats. A: effect on food intake of Ex-4 (5 μg/kg ip) in 24-h-fasted Sprague-Dawley rats (saline, n = 10; Ex-4, n = 6). B: glucose levels following administration of Ex-4 (5 μg/kg ip) in overnight-fasted, conscious Long-Evans rats (sal, n = 7; Ex-4, n = 9). C: glucose levels following iv administration of several doses of Ex-4 (1, 5, and 20 μg/kg) combined with IPGTT (1 g/kg) in ad libitum-fed anesthetized Sprague-Dawley rats (n = 7). Values are expressed as means ± SE. *P < 0.05, **P < 0.01, and ***P < 0.001 vs. saline-treated control group. Two-way ANOVA for multiple comparisons.

Fig. 3. Central administration of Ex-4 increases blood glucose levels in conscious rats. Effect of intracerebroventricular (icv) administration of Ex-4 (0.1, 1, and 5 μg) after 2 h on blood glucose levels of conscious, 24-h-fasted Sprague-Dawley rats (n = 9–12). Values are expressed as means ± SE. ***P < 0.001 vs. saline-infused control group, 1-way ANOVA.
levels after 30 min (Fig. 4D). Interestingly, the increase in blood glucose levels induced by Ex-4 was enhanced significantly in vagotomized rats. These data demonstrate that hyperglycemia induced by Ex-4 is not caused directly by vagal signals, although there seems to be some influence of the parasympathetic nervous system (PNS) to mitigate this response.

Pretreatment with HXM alone to block the ANS did not affect either basal glucose or insulin concentrations (Fig. 4E–H) but attenuated Ex-4-induced hyperglycemia in rats with or without a glucose challenge (Fig. 4, E and G). Notably, Ex-4 induced a discrete but significant early increase in insulin secretion in the absence of any hyperglycemia (Fig. 4F). This increase of insulin secretion was not blocked but rather enhanced significantly by the pretreatment with HXM, even in conditions of GTT (Fig. 4, F and H). These results confirm our previous observation that a fully functional ANS is essential to induce the acute increase of blood glucose after the peripheral administration of Ex-4. However, there is an insulinotropic effect of Ex-4 that is apparent soon after administration, and this effect is independent of hyperglycemia and enhanced by the ANS blockade.

To study whether the catecholamines secreted by the adrenal medulla after the activation of the SNS could be responsible for the acute hyperglycemia, we administered Ex-4 in overnight-fasted rats that had previously undergone adrenal medullectomy. Ex-4 injection failed to induce hyperglycemia in medullectomized rats (Fig. 5A). The integrity of the adrenal cortex in these rats was demonstrated by postmortem histological analysis of the adrenal glands (data not shown) and by the increase in corticosterone induced by Ex-4 (Fig. 5B) as a result of the activation of the hypothalamic-pituitary-adrenal axis. Ex-4 increased urinary content of MN and NMN in sham-operated rats. Adrenal medullectomy prevented the increase of urinary MN, but not NMN, induced by Ex 4 (Fig. 5, C and D). Plasma glucagon levels 30 min after ip administration of Ex-4 or saline did not differ (Fig. 5E). These findings indicate that Ex-4-induced hyperglycemia is independent of glucocorticoid secretion or the direct effects of norepinephrine secreted by SNS. 

Fig. 4. Ex-4 increases blood glucose levels through the sympathetic branch of the autonomous nervous system. A: glucose levels 1 h following ip administration of Ex-4 (5 μg/kg) in 72-h-fasted conscious Sprague-Dawley rats pretreated with the ganglionic nicotinic blocker hexamethonium (HXM) (30 mg/kg at −30 min; n = 6). B: glucose levels 1 h following icv administration of Ex-4 (1 μg) in 72-h-fasted conscious Sprague-Dawley rats pretreated with HXM (30 mg/kg at −30 min; n = 6). C: glucose levels 1 h following ip administration of Ex-4 (5 μg/kg) in 48-h-fasted conscious Sprague-Dawley rats pretreated with the inhibitor of the norepinephrine release guanethidine (GU) (30 mg/kg at −30 min; n = 6). D: glucose levels following ip administration of Ex-4 (20 μg/kg) in 72-h-fasted anesthetized Sprague-Dawley rats, previously vagotomy (Vx) at cervical level (−20 min; n = 8). Glucose (E) and insulin levels (F) following intravenous administration of Ex-4 (5 μg/kg) in 48-h-fasted anesthetized Sprague-Dawley rats pretreated with HXM (30 mg/kg, −30 min; n = 8). Glucose (G) and insulin levels (H) following iv administration of Ex-4 (5 μg/kg) combined with d-glucose administration (1.5 g/kg ip) in ad libitum-fed Sprague-Dawley rats pretreated with HXM (30 mg/kg, −30 min; n = 7–8). Values are expressed as means ± SE. *P < 0.05, **P < 0.01, and ***P < 0.001 vs. saline-infused control group. #P < 0.05, ##P < 0.01, and ###P < 0.001 vs. nonpretreated (E and F), sham-operated Ex-4-injected group (D), or time 0 min (H). One- (A–C) or two-way ANOVA (D–F), Bonferroni post hoc test.
efferents but highly dependent on epinephrine released from the adrenal medulla.

**Waning of the Hyperglycemic Effect of Ex-4 After Chronic Administration**

To determine whether the hyperglycemic effect of Ex-4 persists over time, glucose levels were measured after an acute challenge with ip Ex-4 (15 μg/kg) in Wistar rats that had been pretreated with either Ex-4 (10 μg/kg ip) or saline daily for 14 days. Ex-4 induced a significant increase in glucose levels for ≤6 h in animals chronically exposed to saline or Ex-4 administrations (Fig. 6). However, this increase was significantly lower in rats that were previously pretreated with Ex-4 compared with saline-pretreated rats, suggesting that Ex-4-induced hyperglycemia is a phenomenon that is susceptible to adaptation.

To confirm the apparent adaptation to the hyperglycemic effect of Ex-4, we infused Ex-4 (15 μg/d) or saline to Long-Evans rats for 7 days using osmotic minipumps. Rats infused with Ex-4 showed a significant decrease of food intake during the first dark phase (Fig. 7A). Thereafter, the saline-treated rats were pair-fed the same amount of food that was eaten by the Ex-4-treated animals (Fig. 7B) so that body weight and fat mass were comparable in both groups (Fig. 7, C and D). Consistent with our other observations, Ex-4 induced a significant acute increase in blood glucose levels seen 3 and 6 h after the start of treatment (Fig. 7E). However, after 1 day of treatment both the Ex-4- and saline-treated rats showed similar levels of glycemia (Fig. 7F). These results indicate that although the increase in blood glucose induced by Ex-4 is robust, it becomes attenuated with continuous exposure to the peptide. After 7 days of infusion, an IPGTT was performed in overnight-fasted Ex-4-treated and control rats. Glucose levels showed a strong trend to be lower in the Ex-4-treated rats (Fig. 7G). Although C-peptide levels did not differ statistically from the control group (data not shown), when corrected for differences in glycemia an insulinotropic effect of Ex-4 in treated rats was apparent (Fig. 7H).

**DISCUSSION**

Ex-4 combines robust GLP-1R agonism with resistance to degradation by DPP IV, making it a potent antidiabetic agent. The effects of Ex-4 to reduce blood glucose have been demonstrated in animal models of diabetes (38, 39, 42) and in diabetic patients. However, in contrast to these chronic effects of Ex-4 to promote glucose metabolism, the present set of experiments demonstrate that in rats Ex-4 induces an acute effect to increase blood glucose. Ex-4-induced hyperglycemia was dose dependent, developed 15–30 min after peptide administration independent of insulin secretion, and mediated by the GLP-1R. The doses of Ex-4 that cause this response are certainly pharmacological but include doses in the range that...
has been used to demonstrate chronic benefits on glucose tolerance in both rats and mice (15, 25). The acute effect of Ex-4 to raise blood glucose was observed with either peripheral or CNS administration of peptide and was abolished with sympathetic, but not parasympathetic, blockade and by adrenal medullectomy. These findings indicate that acute administration of Ex-4 activates the sympathetic nervous system and that this response is sufficient to cause hyperglycemia, even in the presence of augmented early insulin secretion. This novel set of observations indicates that the neural activity of Ex-4 in rats is complex and not uniformly protective of glucose homeostasis.

It is now well established that the GLP-1R is expressed in the peripheral and central nervous systems (20, 21, 23, 29, 33, 34, 37, 41) and can mediate a range of behavioral and metabolic effects. Recent findings suggest that some neural activation through GLP-1R signaling promotes glucose metabolism (23, 33, 37) by suppressing hepatic glucose production, increasing hepatic glucose uptake, and enhancing insulin secretion. However, the role of brain GLP-1R signaling on glucose metabolism is complex since chronic central administration of a GLP-1R antagonist seems to have beneficial effects in mice fed a high-fat diet (22). Ex-4 activates the SNS when given peripherally or centrally to mice (40, 41) and causes pressor and tachycardic responses in rats also through SNS activation (4, 5, 13, 14). Our results are consistent with these earlier findings in that an increase in SNS activity of sufficient magnitude to raise blood pressure could also increase blood glucose likely through enhanced hepatic glucose production. The sympathetic activation triggered by Ex-4 causes hyperglycemia independent of steady-state fuel availability, since the glucose increase occurs in rats fed ad libitum or given ip glucose as well as in rats after overnight and prolonged fasting. This effect of Ex-4 presents an important confounder of studies involving acute administration of the peptide in rat models. Although acute administration of Ex-4 has been shown to stimulate insulin secretion and ameliorate hyperglycemia in animals, most of these demonstrations have been in mice (17, 42). In rats, the acute glycemic effects of Ex-4 are less clear. Parkes et al. (30) reported that iv Ex-4 caused a dose-dependent increase of insulin in anesthetized Lewis rats, without a significant effect on iv glucose tolerance, although both the treated and control animals in this experiment were relatively hyperglycemic with fasting values >10 mM. Similarly, Fran-
gioudakis et al. (12) demonstrated that iv Ex-4 enhanced glucose-stimulated insulin secretion in anesthetized and conscious Wistar rats without significant differences in blood glucose compared with controls. These studies used Ex-4 doses of ≤10 μg/kg, comparable with those used in our experiments. Although they did not note an acute hyperglycemic effect of Ex-4, it is worth noting that their studies were relatively short, and they did not assess glucose across the time periods where we saw the most pronounced effects. However, several other groups have observed that acute administration of Ex-4 causes hyperglycemia. Chronic administration of Ex-4 subcutaneously, in doses similar to what were used in the present studies, increased blood glucose, ACTH, corticosterone, and catecholamines in diabetic rats (27). In addition, Aziz and colleagues (2, 3) gave Ex-4 ip in doses of 1.5 μg/kg to fasted Wistar rats and noted hyperglycemia compared with saline-treated controls, an effect that was potentiated by protein ingestion. Thus, although there is a paucity of reports on acute effects of Ex-4 on glucose metabolism in rats, there exist data that are compatible with the findings reported here.

Although hyperglycemia was prevented by the blockade of both branches of the ANS with HXM, this treatment actually enhanced insulin release stimulated by peripheral Ex-4. This likely reflects a reduction of catecholaminergic signaling in the pancreatic islets, since the catecholamines inhibit insulin secretion (32). The parasympathetic branch of the ANS (PNS) does not seem to contribute to the acute hyperglycemic effect of Ex-4. Rather, cervical vagotomy significantly enhanced Ex-4-induced hyperglycemia, suggesting that parasympathetic signaling provides some counterbalance against Ex-4-induced activation of the SNS. The effects of both the SNS and PNS are blocked by HXM, but specific pharmacological blockade of the sympathetic branch of the ANS by GU and adrenal medullectomy ameliorated Ex-4-mediated hyperglycemia. GU inhibits the release of norepinephrine, present in the presynaptic terminals of the SNS, including those innervating the adrenal medulla, which seems to be the critical mediator of Ex-4-induced hyperglycemia.

The administration of Ex-4 and GLP-1 in our studies was insulinotropic and lowered the glycemic response to iv glucose, at least in the early phases of the glucose tolerance tests. In contrast to Ex-4, GLP-1 did not trigger any later rise in blood glucose relative to control animals. However, since the hyperglycemia induced by Ex-4 was completely abolished by pretreatment with Ex-9, it seems likely that both peptides were acting through the GLP-1R. The differential effect of GLP-1 and Ex-4 cannot be explained by DPP IV metabolism of the former, since the addition of vildagliptin did not change the response to GLP-1. Although we cannot explain the disparate effects of these two GLP-1R agonists to cause acute activation of the SNS, we have recently reported differential effects of Ex-4 and GLP-1 to cause anorexia that are also metabolism independent (6).

Our data indicate that SNS stimulation by Ex-4 must wane with repeated exposure to the peptide because the hyperglycemic effect diminishes over time. It is notable that chronic exposure to Ex-4 tended to improve glucose tolerance, consistent with the many studies showing beneficial effects on glycemic control in chronic studies in rats (15, 26, 42). Disappearance of the hyperglycemic effect of Ex-4 with chronic administration suggests that the sympathetic mechanisms activated by Ex-4 are susceptible to the development of adaptation or desensitization with protracted exposure to the compound.

Previous reports in animal models show that GLP-1R signaling in the CNS mediates visceral illness (34) and the response to stress (20). Consistent with this, nausea and vomiting are the most common adverse effects of Ex-4 therapy in diabetic patients (1). There is little evidence for acute SNS activation by synthetic Ex-4 in humans, but this has not been a specific focus of research in this area. However, iv administration of Ex-4 to healthy volunteers did not affect heart rate or blood pressure (9). Nonetheless, SNS activation can inhibit gastrointestinal motility acutely and could contribute to the well-known side effects of Ex-4. Interestingly, these side effects reported in humans given Ex-4 disappear after the initial days of treatment (11, 18). Our findings taken in the context of these clinical observations raise the possibility of short-term SNS activation with pharmacological doses of Ex-4.

In summary, we have demonstrated a novel aspect of neural activation by Ex-4, namely hyperglycemia, an effect that was robust and demonstrable across a range of experimental settings in three commonly used strains of rats. Our results demonstrate a response to GLP-1R activation that appears to be specific for Ex-4, is mediated by the SNS, and raises blood glucose even in the face of potentiated insulin secretion. Since the effects we have demonstrated are the result of pharmacological stimulation of the GLP-1R, it seems unlikely that activation of the SNS is a usual component of regulation by endogenous GLP-1. Our findings raise important considerations for investigators using Ex-4 in studies of rats and support direct testing of whether effects derived from the activation of the SNS are also seen in other species and whether those effects could be related to adverse effects of drugs with GLP-1R activity.

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DISCLOSURES

No conflicts of interest are declared by the author(s).

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