Protection of Glucagon-Like Peptide-1 in Cisplatin-Induced Renal Injury Elucidates Gut-Kidney Connection

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ABSTRACT

Accumulating evidence of the beyond-glucose lowering effects of a gut-released hormone, glucagon-like peptide-1 (GLP-1), has been reported in the context of remote organ connections of the cardiovascular system. Specifically, GLP-1 appears to prevent apoptosis, and inhibition of dipeptidyl peptidase-4 (DPP-4), which cleaves GLP-1, is renoprotective in rodent ischemia-reperfusion injury models. Whether this renoprotection involves enhanced GLP-1 signaling is unclear, however, because DPP-4 cleaves other molecules as well. Thus, we investigated whether modulation of GLP-1 signaling attenuates cisplatin (CP)-induced AKI. Mice injected with 15 mg/kg CP had increased BUN and serum creatinine and CP caused remarkable pathologic renal injury, including tubular necrosis. Apoptosis was also detected in the tubular epithelial cells of CP-treated mice using immunoassays for single-stranded DNA and activated caspase-3. Treatment with a DPP-4 inhibitor, alogliptin (AG), significantly reduced CP-induced renal injury and reduced the renal mRNA expression ratios of Bax/Bcl-2 and Bim/Bcl-2. AG treatment increased the blood levels of GLP-1, but reversed the CP-induced increase in the levels of other DPP-4 substrates such as stromal cell–derived factor-1 and neuropeptide Y. Furthermore, the GLP-1 receptor agonist exendin-4 reduced CP-induced renal injury and apoptosis, and suppression of renal GLP-1 receptor expression in vivo by small interfering RNA reversed the renoprotective effects of AG. These data suggest that enhancing GLP-1 signaling ameliorates CP-induced AKI via antiapoptotic effects and that this gut-kidney axis could be a new therapeutic target in AKI.


AKI is a common clinical complication. Despite supportive care including renal replacement therapy, the 5-year mortality rate after AKI remains high.1 Apoptotic cell death is a prominent and characteristic feature of AKI, especially when caused by nephrotoxic medications or contrast agents.2 Cisplatin (CP) is an extremely effective chemotherapeutic agent, but its acute nephrotoxicity often limits its applicability.3 The exposure of tubular epithelial cells to CP reportedly activates complex signaling pathways that engender cell injury and death. Among those pathways, the apoptotic pathway has been investigated. The intrinsic mitochondrial pathway involving several regulatory factors such as Bax and Bcl-2 has been elucidated.4 Glucagon-like peptide-1 (GLP-1), an incretin hormone, is released from the gut in response to fat or carbohydrate intake. It contributes to negative feedback control of blood glucose by stimulating

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insulin secretion, inhibiting glucagon, and slowing gastric emptying.\textsuperscript{5} GLP-1 receptor (GLP-1R), a member of the glucagon receptor family of G protein–coupled receptors, is present in the pancreas, brain, heart, blood vessels, and kidney.\textsuperscript{6–9} Kim et al. recently reported a link between GLP-1 release from the gut and the atrial natriuretic peptide (ANP) excretion from the heart.\textsuperscript{10} In the kidney, GLP-1R reportedly expresses mainly at proximal tubules in rats, porcine, and humans.\textsuperscript{11,12} GLP-1 signaling through GLP-1R reportedly enhances cAMP as a second messenger.\textsuperscript{13} Activated GLP-1R stimulates the adenylyl cyclase pathway, which results in increased insulin synthesis as well as insulin release. Meanwhile, the potential antiapoptotic effect of GLP-1 has been demonstrated in diabetic rodents and islet cell lines.\textsuperscript{14–17}

GLP-1 actually has a $t_{1/2}$ of $<3$ minutes in the circulation because of rapid cleaving by the widely expressed enzyme dipeptidyl peptidase-4 (DPP-4, also known as CD26).\textsuperscript{18,19} Therefore, therapeutic manipulation of the GLP-1 system includes strategies that inhibit the degradation of bioactive serum GLP-1 by the DPP-4 inhibitor, or that uphold GLP-1 pathway activity with long-acting GLP-1R agonists. Glorie et al. recently reported renal protection of DPP-4 inhibitors in rodent ischemia-reperfusion injury models.\textsuperscript{20} However, it remains uncertain whether the GLP-1–mediated pathway plays a crucial role in renal protection because DPP-4 cleaves other molecules such as stromal cell–derived factor-1 (SDF-1) and neuropeptide Y (NPY).\textsuperscript{5,21}

Using a mouse CP-induced AKI (CP-AKI) model, we investigated the protective effect of a DPP-4 inhibitor alogliptin (AG), which has been clinically available. We evaluated mainly antiapoptotic effects as a possible mechanism of action. In addition, we demonstrated that no other molecules can be degraded by DPP-4, but that GLP-1 had an important role in renal protection by administering a GLP-1R agonist to the CP-AKI model.

RESULTS

Alogliptin Reduces Renal Dysfunction, Pathologic Injury, and Oxidant Stress in CP-Induced AKI
Male C57BL/6 mice aged 7–8 weeks were given 10 mg/kg body weight of AG or vehicle (saline) orally once daily for 7 days with a subsequent intraperitoneal administration of 15 mg/kg of CP. AG treatment was continued every 24 hours until the animals were euthanized at 96 hours after CP injection. Figure 1A presents the time course of BUN in mice. Remarkably increased BUN was found at 72 hours and 96 hours after CP injection, but was attenuated significantly by AG treatment, which also inhibited serum creatinine elevation after CP administration (Figure 1B). No significant difference due to AG treatment was found in body weight or blood glucose (Figure 1, C and D). Pathologic analysis revealed that renal tubular necrosis accompanied by brush border loss and cast formation was induced 96 hours after CP injection (Figure 2A). These changes were significantly attenuated in AG-treated mice. Semiquantitative assessment of histologic damage demonstrated significant protection by AG in CP-induced kidney injury (Figure 2B). We also evaluated the delayed treatment of AG in CP-induced kidney injury. The animals received AG 6 hours later from CP injection and AG treatment was continued daily until they were euthanized. Delayed AG treatment also showed renal protection, with significantly lower BUN (96 hours) and serum creatinine (72 hours and 96 hours) in the delayed treatment group compared with the untreated group (Figure 1, E and F).

Oxidative stress was evaluated by immunohistochemistry of 4-hydroxy-nonenal (4-HHE) and 8-hydroxydeoxyguanosine...
Reportedly, these oxidative stress markers were found in renal ischemia-reperfusion or CP-AKI models.\(^22,23\) In this study, accumulation of 4-HHE and 8-OHdG was detected in renal tubular epithelial cells in CP-injected animals. AG treatment remarkably reduced positive areas of 4-HHE and 8-OHdG (Figure 2C). Quantitative analysis of 4-HHE– and 8-OHdG–positive areas confirmed the reduction of oxidative stress by AG treatment (Figure 2D).

**AG Attenuates Renal Apoptosis in CP-Induced AKI**

Renal tubular apoptosis was evaluated using two different well-established early apoptosis indicators: single-stranded DNA (ssDNA) and cleaved caspase-3. At 96 hours after CP injection, remarkable positive staining of ssDNA and cleaved caspase-3 was found in renal tubular cells (Figure 3A). Quantitative real-time PCR analysis revealed that mRNA expression ratios of proapoptosis to antiapoptosis increased at 96 hours after CP injection. The AG treatment improved these mRNA ratios (Figure 3, C–F).

**Activity of DPP-4 and Substrates of DPP-4 in CP-AKI**

DPP-4 activity was evaluated in plasma and kidney homogenates. Before and 96 hours after CP administration, plasma and renal DPP-4 activity was significantly suppressed by AG treatment (Figure 4, A and B). Substrates of DPP-4 in blood, including GLP-1, SDF-1, and NPY, were measured using ELISA. Although pretreatment of AG did not change the plasma GLP-1 levels, administration of AG significantly increased plasma GLP-1 at 48 hours after CP administration (Figure 4C). In contrast to GLP-1, AG-untreated animals showed higher plasma levels of SDF-1 and NPY than AG-treated mice (Figure 4, D and E).

**Treatment of GLP-1 Receptor Agonists in CP-AKI**

To clarify the role of GLP-1 in CP-AKI, we administered GLP-1R agonist exendin-4 (Ex-4) to CP-injected mice. GLP-1R expression in the normal kidney was confirmed using RT-PCR. CP injection and administration of AG and Ex-4 did not change the expression levels (Figure 5A). Ex-4 significantly reduced BUN and serum creatinine and attenuated pathologic injury such as tubular necrosis induced by CP injection (Figure 5, B–D). Similarly to the antiapoptotic effects observed in AG, Ex-4 treatment reduced renal tubular cell apoptosis as evaluated by ssDNA and caspase-3 immunostaining (Figure 6, A and B). Quantitative RT-PCR analysis revealed that Ex-4 treatment decreased mRNA expression ratios of proapoptosis to antiapoptotic regulators (Figure 6, C–F).
Renal Expression of GLP-1Rs and In Vivo Treatment by Small Interfering RNA in CP-AKI

GLP-1R expression in the kidney was confirmed by RT-PCR (Figure 5A) and Western blot analysis (Figure 7A). Localization of GLP-1R in proximal tubular cells was demonstrated by immunohistochemistry (Figure 7A). To demonstrate that the GLP-1R-mediated pathway contributes to renal protection by AG, we conducted an experiment using in vivo siRNA against GLP-1R. First, animals received either 100 μg of scrambled small interfering RNA (siRNA) (negative control) or GLP-1R siRNA at 0 and 48 hours after CP injection. We then harvested kidneys at 96 hours. The GLP-1R knockdown efficacy in the kidney was confirmed using quantitative real-time RT-PCR and Western blot analysis (Figure 7, B and C). Suppressing GLP-1R expression by in vivo siRNA cancelled the renal-protective effect of AG, which was evaluated using BUN, serum creatinine, and pathologic analysis (Figure 7, D–F).

**DISCUSSION**

This study demonstrated protective effects of DPP-4 inhibition on CP-AKI. AG pretreatment for 1 week attenuated renal dysfunction and pathologic injury, including acute tubular necrosis with accumulation of oxidative stress. Delayed treatment started at 6 hours after CP injection also significantly reduced BUN and serum creatinine. Tubular cell apoptosis was also significantly reduced with evidence of upregulation of antiapoptotic regulator expression. Plasma GLP-1 levels in AG-treated animals were significantly higher than in untreated animals, although other DPP-4 substrates such as SDF-1 and NPY were decreased by AG treatment. In addition, treatment with Ex-4, a GLP-1R agonist, also attenuated CP-AKI with moderation of apoptosis in tubular cells. Finally, suppressing GLP-1R in the kidney by in vivo siRNA reversed the renal protection of AG in CP-AKI. These data suggest that GLP-1–mediated antiapoptotic effects attenuated CP-AKI via the gut-kidney connection.

DPP-4, also known as CD26, is a 110-kDa ectoenzyme that belongs to the serine protease family.21,24,25 DPP-4 is widely distributed on the cell surface of endothelial and epithelial cells. In the kidney, DPP-4 is reportedly expressed mainly on the brush border of proximal tubular cells and in interstitial and glomerular cells. Infiltrating immune cells in the renal ischemia-reperfusion injury model also express DPP-4 because DPP-4 degrades proline-containing or alanine-containing peptides21 and might therefore have various possible pleiotropic effects.28 DPP-4 physiologically cleaves cytokines, chemokines, and neuromodulators involved in inflammation, immunity, and vascular function. Among them,
GLP-1, SDF-1, and NPY are well-known endogenous physiologic substrates for DPP-4.5 SDF-1, reportedly expressed in the kidney after ischemia-reperfusion injury, is expected to protect the kidney by mobilizing progenitor cells from the bone marrow.29 However, its functional role in AKI remains uncertain.30,31 Zaruba et al. reported that pharmacologic inhibition of DPP-4 was able to increase the homing of SDF-1 receptor–positive endothelial progenitor cells at sites of myocardial damage in mice, thereby reducing cardiac remodeling, functional improvement, and survival.32 However, plasma SDF-1 levels in mice treated by the DPP-4 inhibitor were lower than in untreated mice, indicating that plasma SDF-1 might play no role in renal protection by AG. As described above, the SDF-1–CXCR4 pathway mediates migration of resting hematopoietic progenitors. However, the SDF-1–CXCR4 pathway is also suggested to be involved in T lymphocyte migration.33 Because T lymphocytes reportedly contribute to the pathogenesis of CP-induced renal injury,34 reducing the effect of SDF-1 on CXCR4 might attenuate T lymphocyte infiltration and engender renal protection. NPY, another DPP-4 substrate,35 exerts various physiologic actions, including central regulation of food intake and regulation of the cardiovascular and the immune systems. Previous studies report that circulating NPY is substantially elevated according to the level of kidney function.36,37 In this study, plasma NPY in AG-treated mice was lower than in untreated mice, similarly to SDF-1. Therefore, we concluded that NPY did not contribute to renal protection by AG.

In contrast to SDF-1 and NPY, AG upheld the gut-released hormone, GLP-1, at a significantly higher level than in untreated mice. These data support the hypothesis that AG attenuated CP-AKI by increasing the GLP-1 levels. As a further demonstration, we conducted another experiment in which GLP-1R agonist Ex-4 was administered to CP-AKI. Ex-4 similarly attenuated CP-AKI to AG. Furthermore, suppressing GLP-1R expression by in vivo siRNA exacerbates CP-AKI even under the treatment of AG. Therefore, increased blood GLP-1 concentrations demonstrate the renal-protective role of AG in CP-AKI, at least in part.

Apoptosis is a crucial mechanism in the pathophysiology of CP-AKI.4 Along with the improvement in renal function (BUN and serum creatinine) and pathologic injury (tubular necrosis and oxidative stress), tubular epithelial cell apoptosis in CP-AKI was significantly attenuated by AG and Ex-4. We further examined proapoptosis and antiapoptosis regulator protein expression (Bax and Bim versus Bcl-2 and Bcl-xL). The balance between proapoptotic and antiapoptotic family members determines whether a cell will undergo apoptosis. In the mouse CP-AKI model, Bax in active renal tubular cells accumulated in the mitochondria.38 In vitro analysis of CP-induced renal tubular cell apoptosis revealed that antiapoptotic regulators such as Bcl-2 and Bcl-xL prevent cell death mainly by sequestering proapoptotic protein Bim.39,40 This study showed significantly decreased ratios of proapoptosis to antiapoptotic regulators by DPP-4 inhibition and GLP-1R agonist injection.

Activation of GLP-1R was reported to inhibit apoptosis with increased expression of Bcl-2 and Bcl-xL in a cultured mouse insulinoma cell line.15 DPP-4 inhibition or GLP-1R agonist prevented β cell from apoptosis in the mouse model using fatty acid administration.41 Mice with a targeted disruption of the GLP-1R gene (GLP-1R−/− mice) exhibit increased susceptibility to streptozotocin–induced β cell apoptosis.16 GLP-1 also reportedly promotes the expression of antiapoptotic protein Bcl-2 and reduces proapoptotic caspases in mouse.
embryonic stem cells. Moreover, GLP-1 treatment in combination with a DPP-4 inhibitor diminishes activation of the proapoptotic protein Bad and reduces the infarct size in isolated perfused rat heart models of ischemia-reperfusion. These observations sustain the inference that GLP-1 exerts antiapoptotic effects in CP-AKI.

Several reports describe involvement of the GLP–mediated pathway in renal physiology, such as in hemodynamics and salt handling or proliferation of human mesangial cells. Limei et al. reported that DPP-4 inhibitor sitagliptin administered to hypertensive rats spontaneously improved endothelial function by restoring nitric oxide bioavailability. Glorie et al. reported that DPP-4 inhibitor vildagliptin attenuated AKI in a mouse ischemia–reperfusion model. Although the mechanisms of action of DPP-4 inhibition on ischemic AKI were not clarified in the above-mentioned reports, we clarified for the first time that a GLP-1–mediated antiapoptotic effect contributed to renal protection in a mouse CP-AKI model. More recently, Kim et al. reported that GLP-1 regulates ANP release from the heart and thus controls BP. The renoprotective effect of GLP-1 in this study might have some relation to the previously reported renoprotection by ANP. It remains uncertain whether DPP-4 inhibition and GLP-1 pathway mediation interfere with the antitumor effects of CP. Additional experiments must be conducted before our findings can be translated to clinical use.

In conclusion, DPP-4 inhibition by AG attenuated mouse CP-AKI. Among the potential substrates of DPP-4, GLP-1 contributes to renal protection via suppression of tubular cell apoptosis. Results obtained using experiments with GLP-1 agonist Ex-4 and siRNA to GLP-1R clearly demonstrated the involvement of GLP-1 in the renal dysfunction of CP-AKI. Considering that GLP-1 is the gut-derived protein, our data provide new insight of the gut-kidney axis into the disease mechanism. Therefore, the GLP-1–mediated pathway is anticipated as a new drug target in AKI.

**CONCISE METHODS**

**Experimental Animal Model**

Male C57BL/6 mice aged 7–8 weeks (Japan SLC Inc., Hamamatsu, Japan) were kept on a 12-hour light/dark cycle with free access to diet and water. All animal experiments were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the University of Tokyo Institutional Review Board.

Animals were orally given 10 mg/kg body weight of selective DPP-4 inhibitor AG (Takeda Pharmaceutical Co Ltd, Tokyo, Japan) or vehicle once daily from 7 days before to 96 hours after CP. In a delayed treatment experiment, AG administration with the same dose was started from 6 hours after CP injection until mice were euthanized (96 hours). In another set of experiments, 100 pmol/kg of GLP-1R agonist Ex-4 (Bachem, Bubendorf, Switzerland) was given once daily via an intraperitoneal injection from 7 days before to 96 hours after CP injection.

We administered a single intraperitoneal injection of 15 mg/kg body weight of CP (Nippon Kayaku, Tokyo, Japan) or saline. Blood was collected serially at each 24-hour interval after CP injection. Mice were euthanized at 96 hours after CP injection, at which time their kidneys were harvested for analyses.

GLP-1R siRNA was obtained from Invitrogen (Carlsbad, CA) as annealed, presigned in vivo siRNA (sequence 5′-GGAACACUCUCAACCGCUACUGU-3′, sense). Scrambled siRNA was used as negative control siRNA. A 100 μg dose of GLP-1R siRNA or scrambled siRNA was injected intravenously twice, at 0 hours and 48 hours after CP injection. The GLP-1R knockdown efficacy was determined at 96 hours using either quantitative real-time RT-PCR or Western blot analysis (see below).
Measurement of BUN and Serum Creatinine

BUN was measured using the urease indophenol method (Wako Pure Chemical Industries Ltd, Osaka, Japan) with an absorbance 96-well plate reader (Molecular Devices Corp, Sunnyvale, CA) at a wavelength of 570 nm. Serum creatinine was measured using high-performance liquid chromatography, as described previously.51

Morphologic Evaluation of Kidneys

For histologic examination, the kidneys were resected from mice after perfusion with PBS. Formalin-fixed sections were stained with periodic acid–Schiff. Morphologic evaluation of acute tubular necrosis was performed using established criteria in a blinded manner.52,53

Immunohistochemical Staining

Immunohistochemical staining of 3-µm paraffin sections was performed using an indirect immunohistochemical technique. After deparaffinization, the nonspecific reaction for horseradish peroxidase was blocked using 3% hydrogen peroxide in methyl alcohol for 20 minutes. Antigen retrieval procedures using microwaves (H2800; Energy Beam Sciences, East Granby, CT) were necessary for all staining. Specimens were blocked using the mouse IgG blocking reagent (Vector Laboratories Inc, Burlingame, CA) for immunohistochemical staining with mice mAbs. A primary polyclonal rabbit anti-ssDNA antibody (DakoCytomation, Glostrup, Denmark), cleaved caspase-3 antibody (Cell Signaling Technology Inc, Danvers, MA), monoclonal anti-HHE, anti-8-OHdG antibody (NOF Corp., Tokyo, Japan), or polyclonal rabbit anti-GLP-1R (Acris Antibodies GmbH, Herford, Germany) was applied to sections and incubated overnight at 4°C. Subsequent procedures for sections were followed using an ABC system (Vector Laboratories Inc), according to the manufacturer’s protocol. We used 3,3′-diaminobenzidine tetrahydrochloride (Nichirei Corp, Tokyo, Japan) for the substrate–chromogen reaction. These sections were counterstained with hematoxylin. All of these staining procedures were conducted using light microscopy. Images were captured using a charge-coupled device (Nikon Corp., Tokyo, Japan). The quantities of ssDNA– and caspase–3–positive tubular epithelial cells were determined in 10 randomly selected nonoverlapping fields at ×400 magnification in each section of the individual mouse renal cortex. The 8-OHdG– and 4-HHE–positive areas were evaluated using MCID image analysis software (Interfocus Imaging Ltd., Cambridge, UK) in 10 randomly selected nonoverlapping fields at ×400 magnification in each section of the cortex.

Measurement of DPP-4 Activity and Active GLP-1

DPP-4 activity in plasma and whole-kidney homogenates was assessed using an assay kit (DPP-4/CD26; Enzo Life Sciences Inc., Farmingdale, NY) according to a previously reported method.54 The quantification of GLP-1 in the plasma samples was accomplished using a GLP-1 (7–36) amide enzyme immunoassay kit (Millipore Corp, Billerica, MA) that specifically quantifies the biologically active form of GLP-1, GLP-1 (7–36) amide. Mice plasma SDF-1 (R&D Systems, Minneapolis, MN) and plasma NPY (Millipore Corp.) were measured using an enzyme immunoassay kit according to the manufacturers’ protocol. To measure GLP-1, SDF-1, and NPY, an appropriate amount of...
DPP-4 inhibitor (Millipore Corp) was added to each sample immediately after blood collection according to the manufacturer’s instructions.

Measurement of mRNA Expressions by RT-PCR
Total RNA was extracted from whole-kidney homogenates using TRIzol (Invitrogen) 96 hours after injection of CP or saline. A QuantiTect Reverse Transcription Kit (Qiagen Inc, Hilden, Germany) was used to synthesize cDNA from total RNA. For examination of GLP-1R and glyceraldehyde-3-phosphate dehydrogenase expression in the kidney, the amplified product was loaded in an agarose gel (2.0%) and electrophoresis was conducted in Tris-acetate-ethylenediaminetetraacetic acid buffer (TAE, 40mM Tris, 20mM acetic acid, and 1mM EDTA). After electrophoresis, the DNA bands were visualized using ultraviolet transillumination (Fuji film, Tokyo, Japan). Transcripts encoding Bax, Bim, Bcl-2, and Bcl-xL were measured using SYBR Green–based quantitative PCR with Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) and a sequence detection system (Prism 7000; Applied Biosystems), according to the manufacturer’s instructions. The following respective PCR primers were used: Bax (forward, 5′-TTGCTACAGGGTTTCATCCA-3′), Bim (5′-GAAGACCACCCTCAAATGGTTAT-3′), Bcl-2 (5′-CATATTGCTGTCCAGTTCATCTC-3′), GLP-1R (5′-GGGTCTCTGGCTACATAAGGAC-AC-3′), and glyceraldehyde-3-phosphate dehydrogenase (5′-ACCACAGTCCATGCCATTCA-TC-3′ and 5′-TCCACCAGCACGAGGAT-3′).

Western Blot Analyses
Harvested kidneys were homogenized in radioimmunoprecipitation assay buffer (PBS with 0.1% SDS, 0.5% sodium deoxycholate, and 1% nonidet P-40) containing protease inhibitors (1 μg/ml aprotinin, 1 μg/ml phenylmethylsulfonyl fluoride, 0.01 μg/ml leupeptin, and 1 mmol/L sodium orthovanadate). The lysates were separated on a 10% SDS-PAGE gel. After transferring proteins from the gel to a polyvinylidene difluoride membrane (Amerham, Uppsala, Sweden), Western blot analysis was performed using 1:500 diluted anti-GLP-1R antibody and incubated overnight at 4°C (Acris Antibodies GmbH). Subsequently, the chemiluminescent signal labeled using ECL Plus (Amerham Biosciences Corp) was detected.
using ultraviolet transillumination (Fujifilm). The membrane was then incubated in a stripping buffer (2% SDS, 100 mM 2-mercaptoethanol, and 12% 0.5 M Tris-HCl, pH 6.8) to remove all probes. The reprobing procedure was further performed with the antibody to actin (Chemicon, Temecula, CA).

Statistical Analyses
Data are expressed as the mean ± SEM. Differences among experimental groups were determined using one-way ANOVA with post hoc analysis, with calculations performed using JMP 9.0 software (SAS Institute Inc., Cary, NC).

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DISCLOSURES
None.

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