Original Research

Inducible Deletion of UCP2 in Pancreatic β-Cells Enhances Insulin Secretion

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ABSTRACT

In pancreatic β-cells, uncoupling protein 2 (UCP2) is thought to negatively regulate insulin secretion; however, its role is still debated, in part due to the confounding effects of long-term UCP2 deletion in current knockout mouse models. We have now generated an inducible β-cell-specific UCP2 deletion model by crossing loxUCP2 mice with those that express a Cre recombinase-estrogen receptor fusion protein driven by the mouse insulin promoter (MIPCreER). Because tamoxifen, which was used to induce UCP2 deletion, is an uncoupling agent, we initially determined whether tamoxifen affected glycemia. Initially, C57BL/6 control mice were injected intraperitoneally with tamoxifen or vehicle (corn oil [CO]) 3 times in 1 week, and the mice examined 2 weeks postinjection. Both groups of mice displayed similar glucose tolerance and in vivo and in vitro insulin secretion, suggesting no effects of tamoxifen on glucose homeostasis and β-cell function. MIPCreER−loxUCP2 male littermates were then injected with tamoxifen to induce β-cell-specific UCP2 deletion (ind.UCP2BKO) or with CO as described above. UCP2 deletion was confirmed by polymerase chain reaction (PCR) analysis of islets. There were no differences in fasting glucose, glucagon or insulin in ind.UCP2BKO mice and glucose (OGTT) and insulin tolerance tests revealed similar levels of glucose and insulin sensitivity. However, ind.UCP2BKO mice sustained significantly higher plasma insulin levels during an OGTT and isolated islets secreted more insulin in response to high glucose. Together, these results suggest that short-term deletion of β-cell UCP2 in adult mice leads to the direct enhancement of insulin secretion.

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RÉSUMÉ

Dans les cellules β pancréatiques, la protéine découplante 2 (UCP2) régulait négativement l’insulino-sécrétion. Cependant, son rôle est encore controversé, en partie en raison de l’effet de confusion de la délétion de l’UCP2 à long terme dans les modèles actuels de souris knock-out. Nous avons maintenant créé un modèle de délétion inducible de l’UCP2 spécifique des cellules β par le croisement de souris loxUCP2 avec ceux qui expriment une protéine de fusion entre la Cre recombinase et le récepteur des œstrogènes activé par le promoteur d’insuline de la souris (MIPCreER : mouse insulin promoter Cre estrogen receptor). Comme le tamoxtène, qui était utilisé pour induire la délétion de l’UCP2, est un agent découplant, nous avons initialement déterminé si le tamoxtène perturbait la glycémie. Initialement, les souris témoins C57BL/6 ont reçu des injections intrapéritonéales de tamoxtène ou du véhicule (huile de maïs [HM]) 3 fois au cours de 1 semaine, et les souris ont été examinées 2 semaines après l’injection. Les deux groupes de souris ont montré une tolérance au glucose et une insulinosécrétion in vivo et in vitro similaires, ne suggérant aucun effet du tamoxtène sur la homéostasie du glucose et le fonctionnement des cellules β. Les mâles de la portée MIPCreER−loxUCP2 ont reçu une injection de tamoxtène pour induire la délétion de l’UCP2 spécifique des cellules β (ind.UCP2BKO) ou par l’HM comme décrit ci-dessus. La délétion de l’UCP2 a été confirmée par l’analyse PCR des îlots. Il n’y a eu aucune différence dans la glycémie à jeun, le glucagon ou l’insuline chez les souris ind.UCP2BKO et le glucose (TTGO : test de tolérance au glucose par voie orale), et les épreuves de tolérance à l’insuline ont révelé des concentrations similaires de glucose et de sensibilité à l’insuline. Cependant, les souris ind.UCP2BKO ont maintenu des concentrations plasmatiques d’insuline significativement plus élevées durant un TTGO, et les îlots isolés ont sécrété plus d’insuline en réponse au glucose élevé. Ensemble, ces résultats suggèrent que la
Introduction

Uncoupling proteins (UCPs) are a family of transmembrane proteins located in the inner mitochondrial membrane that are generally thought to transport protons, Cl− ions and other monovalent anions (1). Uncoupling protein 2 (UCP2) was discovered by its relatively high sequence homology to UCP1, a classical strong uncoupler that plays a prominent role in thermogenesis (2). Unlike UCP1, the function of UCP2 has been a matter of significant debate over the last decade. Increased UCP2 expression is observed in mouse models of obesity and type 2 diabetes mellitus (3), and in humans polymorphisms in the UCP2 gene have been linked with hyperinsulinism (4) and a UCP2 -866G>A variant with increased risk of type 2 diabetes and obesity (5–8). However, due to the wide tissue expression of UCP2 in metabolically active tissues such as the liver, brain, pancreas and adipose tissue (9), it has been difficult to understand the exact role of UCP2 in β-cell function and how that affects the development of type 2 diabetes. Previous studies have indicated that increased UCP2 activity is negatively associated with insulin secretion. UCP2 control of insulin secretion occurs via uncoupling respiration and limiting adenosine-5′-triphosphate (ATP) production (3,10–13); whereas others contend that UCP2 control of insulin secretion occurs through the modulation of reactive oxygen species (ROS) signals responsible for the amplification of insulin secretory pathway (14–16). In addition UCP2 knockout (3,17) and overexpression (18,19) mouse models have given discrepant results regarding its regulation of insulin secretion making it difficult to understand the exact function of UCP2 in glycemic control and β-cell function.

Much of our understanding of UCP2 function in the β-cell comes from 2 types of mouse models, the whole-body UCP2 knockout model (UCP2−/−) and β-cell specific UCP2 knockout (UCP2BKO) model; however, there are limitations with each of these models. For example, both of these knockout models could experience UCP2 deletion in tissues other than β-cells and deletion occurs during embryogenesis. To create a model for the study of UCP2 in the β-cells that in part overcomes the previous issues, we have generated and characterized a novel β-cell-specific inducible UCP2 knockout mouse model (MIPCreERloxUCP2), β-cell UCP2 deletion was induced by tamoxifen (Tmx), which binds to the Cre-estrogen receptor (CreER) recombinant protein, triggering the translocation of CreER into the nucleus (20), causing specific excision at loxP sites (21) (ind.UCP2BKO model). We have now characterized this model to better understand how β-cell UCP2 contributes to insulin secretion and the control of overall glucose homeostasis. Here, we demonstrate that inducible knockout of UCP2 in the β-cell does not cause glucose or insulin intolerance. Inducible UCP2BKO mice sustained significantly higher plasma insulin levels during an oral glucose tolerance test (OGTT), reflective of their improved ability to secrete insulin. Consistent with this elevated in vivo insulin secretion, ind.UCP2BKO isolated islets secreted more insulin in response to elevated glucose. Taken together, these results suggest that inducible deletion of UCP2 from the β-cell enhances insulin secretion both in vivo and in vitro.

Methods

Animals

Inducible β-cell-specific UCP2 deletion was obtained by crossingloxUCP2 mice (a generous gift from Dr. Bradford Lowell, Harvard University) (16,21) with mice that express the Cre recombinase-estrogen receptor fusion protein driven by the mouse insulin promoter (MIPCreER) (a generous gift from Dr. Lou Philipson, University of Chicago) (22). These MIPCreER mice had been backcrossed to a C57BL/6 background for 5 generations before being crossed to theloxUCP2 mice. Mice were genotyped by standard multiplex polymerase chain reaction (PCR) using ear notch DNA (Fig. 1C). Mice that express the CreER construct and were homozgyous for theloxUCP2 gene were used for experiments. All mice were male and age-matched and maintained on a 129/J-C57BL/6 mixed background. Mice were injected intraperitoneally (i.p.) with either 25 mg/mL Tmx (dissolved in corn oil [CO]) at a concentration of 137.5 mg/kg body weight to induce β-cell-specific UCP2 deletion (ind.UCP2BKO) or with the same volume of CO alone. The mice were injected 3 times, on alternating days. Injections began at 6 week of age (Fig. 1B). To confirm the specificity of Cre expression in β-cells the MIPCreER mice were also crossed to ROSA26eYFP mice. In these mice β-cells express eYFP when Tmx is present to delete theloxP-flanked transcriptional “stop” sequence in ROSA26 locus (23). Similarly, these MIPCreERxROSA26eYFP mice were injected i.p. with either 25 mg/mL Tmx at a concentration of 137.5 mg/kg (dissolved in CO) or with the same volume of CO at 6 weeks of age and islets isolated 2 weeks later. C57BL/6 mice (The Jackson Laboratory, Bar Harbor, ME) were also used to test whether i.p. injection of Tmx had effects on glucose homeostasis and islet insulin secretion. Age-matched 6-week-old male C57BL/6 mice were injected with Tmx or CO in the same way as described above.

Experiments were carried out on MIPCreERloxUCP2 and C57BL/6 mice 2 weeks postinjection (at week 9). Mice were then allowed to recover for 1 week posttolerance tests before being sacrificed for islet isolation and in vitro studies. All animal experiments were approved by the University of Toronto Animal Care Committee, and animals were handled according to the guidelines of the Canadian Council of Animal Care.

Pancratic islet isolation and culture

Mice were anesthetized with 250 mg/kg triclopropane (i.p) injection. The pancreas was perfused via the common bile duct with type-V collagenase (0.8 mg/mL) dissolved in RPMI-1640 solution supplemented with 100 U/mL penicillin, 100 mg/mL streptomycin and 2% bovine serum albumin (BSA). Pancreata were then digested at 37°C in the water bath for 16 to 20 minutes. Islets were mechanically picked from debris tissue and cultured in RPMI-1640 media supplemented with 10% fetal bovine serum, 100 U/mL penicillin and 100 mg/mL streptomycin and were cultured overnight at 37°C before experimentation (14).

Antibodies and immunofluorescent staining

Primary antibodies, were used guinea pig anti-insulin (Thermo Scientific, Waltham, MA), rabbit anti-glucagon, and mouse anti-GFP (that also recognizes eYFP) (New England Biolabs, Ipswich, MA), were used. Secondary antibodies used were cyanine (Cy5)-labeled anti-guinea pig and Cy5-labelled anti-rabbit (Jackson Immuno Research, West Grove, PA) and Alexa 488-fluor-labelled anti-mouse (Invitrogen, Carlsbad, CA) antibodies were also used. Dispersed islet cells plated onto glass coverslips were stained with antibodies as described previously and fluorescence visualized using a laser scanning confocal microscope (Zeiss 510 LSM, Germany) (24).
RNA extraction and reverse transcription of animal tissues

Hypothalamic tissue of ind.UCP2BKO and CO mice was isolated, washed in phosphate buffered saline and stored in RNAlater solutions (Ambion, Austin, TX) at \( -20^\circ C \) until further use. Before extraction, hypothalamic tissues were homogenized using an electronic polytron. Islets of ind.UCP2BKO and CO mice were homogenized manually (vigorously mixed with a pipette) in extraction buffer. Hypothalamic and islet RNA were extracted using an RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Reverse transcription to make cDNA was completed using an M-MLV Reverse Transcriptase Kit (Invitrogen) according to the manufacturer's instructions.

Glucose-stimulated insulin secretion (GSIS)

A total of 8 to 20 islets per condition were washed with ice-cold Krebs Ringer buffer (KRB) (125 mM NaCl, 5.9 mM KCl, 1.28 mM CaCl\(_2\), 5.0 mM NaHCO\(_3\), 25 mM HEPES, and 0.1% (w/v) (BSA) and preincubated at 37°C in KRB plus 2.8 mM glucose for 1 hour. Tubes were put in ice water and the 2.8 mM glucose KRB buffer was replaced with ice-cold 2.8 mM or 16.7 mM glucose KRB and incubated for 30 minutes at 37°C. Insulin secretion was measured using a radioimmunoassay (RIA) kit (Millipore, Billerica, MA) and normalized to islet number.

OGTT

After a 16-hour fast, mice were gavaged with glucose (2 g/kg body weight). Blood glucose was measured at 0, 10, 20, 30, 60 and 120 minutes. Plasma insulin was measured at 0, 10, 20 and 60 minutes using an ultrasensitive insulin ELISA kit (ALPCO Diagnostics, Salem, NH). Plasma glucagon was measured at 0 and 10 minutes by RIA (Millipore).

Intraperitoneal insulin tolerance test

After a 4-hour fast, insulin (1.5 IU/kg body weight) was injected i.p. Blood glucose was measured at 0, 15, 30, 60 and 120 minutes. Plasma glucagon was measured at 0 and 30 minutes using an RIA (Millipore).
that the MIPCreER construct shows Tmx inducible, glucose stimulated insulin secretion. (n=6–8 mice per condition).

Statistical analysis
Statistical significance was assessed by using the Student’s t test (GraphPad Prism 5, La Jolla, CA). A p value of <0.05 was considered significant. All data is expressed as the mean ± standard error (SE).

Results

Knockdown of UCP2 transcripts in β-cells and validation of the mouse model

To assess the specificity of UCP2 knockdown in pancreatic β-cells, standard RT-PCR was performed on islet and hypothalamic RNA. As expected, the deletion of UCP2 exons 3 and 4, including the start codon, was observed in whole ind.UCP2BKO islets (Fig. 1A). Note that the expression of UCP2 in non-β-cells contributes to the detectable UCP2 expression in whole islets, which explains the coexistence of both a wild type and truncated band in ind.UCP2BKO islet sample. A very faint UCP2 deletion band was observed in CO islets, suggesting some aberrant expression of the Cre recombinase transgene in the absence of Tmx. UCP2 deletion was not observed in the hypothalamus, indicating that our novel inducible model has improved β-cell specificity compared to the UCP2BKO model, which showed some deletion of UCP2 in the hypothalamus (21,25) (Fig. 1D). Immunofluorescent staining of dispersed MIP-CreER×ROSA26eYFP islet cells showed YFP protein colocalized with insulin expressing β-cells in the mice injected with Tmx, indicating that the MIPCreER construct shows Tmx inducible, β-cell-specific Cre recombinase. The efficiency of recombination was approximately 75% (Fig. 1E.1). In line with the faint UCP2 deletion band discussed above, very infrequently a YFP-positive, insulin-expressing cell was observed in the CO injected mice (Fig. 1E.2). YFP protein was not localized in glucagon expressing α-cells in ind.UCP2BKO mice suggesting that UCP2 was not deleted from α-cells (Fig. 1E.3,1E.4).

It was reported previously that Tmx, which is a fat-soluble hormone, can affect mitochondrial membrane integrity and effectively uncouple the mitochondrial electron transport chain from efficient ATP synthesis (26). Therefore, we determined whether Tmx alone affected glucose homeostasis and/or insulin secretion. To accomplish this, 6-week-old C57BL/6 black male mice were injected with either Tmx or CO in the same way as the MIP-CreER×loxUCP2 mice (Fig. 1B). After the 2 week recovery period, OGTTs revealed no significant impact of Tmx on either glucose homeostasis or in vivo insulin secretion (n=6–8 mice per condition) (Fig. 2A,B). Additionally, in vitro glucose-stimulated insulin secretion (GSIS) assays indicated no significant difference between islets isolated from Tmx- or CO-injected C57BL/6 mice after exposure to elevated glucose (n=6–8 mice per condition) (Fig. 2C). These results suggest that under the conditions studied the dose and method of Tmx administration alone does not alter glucose homeostasis and islet insulin secretion.

β-cell UCP2 deficiency in adult mice does not affect glucose tolerance or insulin sensitivity

Our previous study using UCP2BKO mice demonstrated that long-term deletion of UCP2 in the β-cell does not alter insulin sensitivity but interestingly, does impair glucose tolerance, possibly due to enlarged α-cell area and enhanced glucagon secretion (16). To determine if these findings were a result of the long-term impact of β-cell UCP2 deficiency or possible ectopic and partial UCP2 deletion, we examined glucose and insulin tolerance in our ind.UCP2BKO model, which is a short-term model of UCP2 deletion in the β-cell. Measurement of body weight revealed no significant difference between ind.UCP2BKO and CO mice (n=10–12 mice per condition) (Fig. 3A). ind.UCP2BKO and CO mice exhibit similar levels of blood glucose, plasma insulin and glucagon after 16 hour fast (n=10–12 mice per condition) (Fig. 3B–D). An OGTT did not disclose any difference in glucose tolerance between ind.UCP2BKO and CO mice (n=10–12 mice per condition) (Fig. 3E). Additionally, ind.UCP2BKO and CO mice also exhibit similar blood glucose after a 4 hour fast and ipITT revealed similar insulin sensitivities (n=12–15 mice per condition) (Fig. 4A,B). Thus, these tolerance tests indicate that in contrast to the long-term UCP2 deficiency in
UCP2BKO mice, short-term β-cell UCP2 deletion does not influence global glucose tolerance or peripheral insulin sensitivity.

β-cell specific UCP2 deficiency alters insulin but not glucagon secretion in vivo

Despite there being no difference in glucose tolerance between the ind.UCP2BKO and CO groups, we looked at the levels of both plasma insulin and plasma glucagon levels during OGTT. The levels of plasma glucagon were similar between ind.UCP2BKO and CO mice (Fig. 3G). Interestingly, however, we found that the ind.UCP2BKO mice had significantly higher plasma insulin levels at 20 and 60 minutes postglucose injection (n=10–12 mice per condition, p<0.05). Because there was no difference in insulin sensitivity between ind.UCP2BKO and CO mice, the elevated plasma insulin of ind.UCP2BKO mice may indicate that the short-term UCP2 deletion in β-cells could cause enhanced in vivo insulin secretion upon the stimulus of elevated blood glucose (Fig. 3F).

UCP2 deficiency in β-cells enhances GSIS in vitro

Our previous study on UCP2BKO mice demonstrated that UCP2 deletion in the β-cell results in improved insulin secretion in vitro (16). Thus, we determined whether higher insulin secretion in vivo also translated into altered secretion in isolated islets. Static insulin secretion assays showed that ind.UCP2BKO and CO had similar basal secretion levels in low glucose condition (2.8 mM), but ind.UCP2BKO islets secreted more insulin per islet compared to CO islets in high glucose (16.7 mM) (n=6 mice per condition, p<0.05) (Fig. 5A). The diameters of ind.UCP2BKO and CO were not significantly different from each other (Fig. 5B) suggesting that changes in islet size were not responsible for enhanced secretion (n=6 mice per condition).

Discussion

The availability of inducible Cre/lox mouse models has facilitated the study of genes of interest with both cell specificity and temporal control. Here we used such a model to delete UCP2 in the β-cells of...
including glucose-sensing hypothalamic neurons, pancreatic islet depletion is problematic because of the wide tissue expression of UCP2, a thermogenic protein that facilitates the generation of ROS signals that are required for amplification of cellular respiration and affect ATP synthesis. Therefore, to eliminate the possibility of Tmx having intracellular effects that negate the deletion of UCP2 we initially examined the effect of Tmx alone on glucose homeostasis and insulin secretion in control mice. No one has investigated whether Tmx injection alone affects whole body glycemia and so this was an important validation experiment. Six-week-old C57BL/6 mice treated with Tmx showed no difference in glucose tolerance compared to CO mice and plasma insulin levels during an OGTT and in vitro GSIS were also similar. These data indicated that the Tmx treatment itself, while causing efficient UCP2 deletion in pancreatic β-cells of ind.UCP2BKO mice, did not alter glucose homeostasis or islet function in control animals and thus validated the use of our model to explore the role of UCP2 in β-cell function. Tmx-injected UC2pBlox/flox mice were originally considered as the control mice for the ind.UCP2BKO mice. However, a previous study has shown that the genetic background can largely influence insulin secretion (17). Therefore it was important to ensure the control mice had the same genetic background as the ind.UCP2BKO mice, and so CO-injected MIPCreER-UCP2Blox/flox mice were deemed the more appropriate control. The experiments using C57BL/6 mice also validate this choice of control.

The previous whole-body UCP2 knockout model (UCP2-/-) (3) and β-cell-specific UCP2 knockout (UCP2BKO) models (16) shed some light on pancreatic β-cell UCP2 function; however, there are fundamental issues with each of these models when studying UCP2 function specifically in the β-cell. The whole-body UCP2-/- model is problematic because of the wide tissue expression of UCP2, including glucose-sensing hypothalamic neurons, pancreatic α-cells and other peripheral glucose sensing tissues (21,24,25). Deletion of UCP2 enhances glucose-induced excitation of certain hypothalamic neurons, including but not restricted to pro-opiomelanocortin (POMC) and melanin-concentrating hormone (MCH) neurons, which could lead to the improved glucose tolerance (21,25). Similarly, deletion of UCP2 from α-cells reduces glucagon secretion under low glucose conditions (24), which also may contribute to improved glycemic control. Therefore, deletion of UCP2 in one or both of these cell types no doubt contributes in part to affect glucose tolerance observed in UCP2-/- mice. Conversely, UCP2BKO mice display glucose intolerance (16); however, this model also has some UCP2 deletion in a population of RIP (rat insulin promoter)-expressing hypothalamic neurons suggesting that deletion in other cells/tissues may have a negative effect on glycemia. Given the limitations of these mouse models, it was necessary to create a β-cell-specific UCP2 deletion model that circumvented most of these limitations. Wicksteed and colleagues have shown that the MIP is the most specific promoter to drive Cre recombination-dependent gene deletion in the β-cells without driving Cre expression in the brain (22). Therefore, the ind.UCP2BKO mouse model was created by crossing MIPCreER mice to loxUCP2 mice and this model allowed more acute deletion of UCP2 in adult mice, without deletion in the hypothalamus (22). The one limitation of this current model that must be acknowledged is that it is accompanied by a low but detectable level of basal nuclear Cre expression in the noninduced state, indicating Tmx-independent Cre recombination and potentially lower β-cell UCP2 expression in control compared to wild type mice (28). Zhang and colleagues showed that UCP2+/− heterozygous mice had higher insulin secretion levels compared to the wild type mice (3), indicating there might be an intermediate phenotype. Therefore, this basal level of deletion in the CO mice might slightly dampen the effects of UCP2 deletion in ind.UCP2BKO mice. Despite this we show even lower expression of UCP2 in the ind.UCP2BKO islets. Using this ind.UCP2BKO model we demonstrate that acute deletion of UCP2 does not affect whole body glycemia over the time period studied, despite an increase in insulin secretory capacity. It is possible that some counter-regulatory response has limited the effect of enhanced insulin secretion to maintain normoglycemia and differences in glycemia might be observed over a longer time period.

Inducible UCP2BKO mice displayed enhanced in vivo and in vitro glucose stimulated insulin secretion. Previous studies have shown different potential mechanisms of UCP2 control of insulin secretion. Some argue that UCP2 uncouples respiration and limits ATP production to suppress insulin secretion: overexpression of UCP2 in rat islets and INS-1 cells decreased ATP content and inhibited GSIS (10,11,28), whereas UCP2 deletion in mouse islets enhanced ATP production and GSIS (3). Other studies suggest that UCP2 serves as a mild uncoupler in β-cells that only influences ROS generation rather than uncoupling respiration/ATP production. Other studies, including ours, using UCP2BKO mice, indicate that UCP2 controls ROS signals that are required for amplification of

Figure 5. Inducible UCP2BKO islets exhibit enhanced glucose stimulated insulin secretion (GSIS). (A) ind.UCP2BKO islets displayed enhanced GSIS compared to CO islets. HG, high glucose (16.7 mM); LG, low glucose (2.8 mM). (B) The islet diameters of ind.UCP2BKO and CO were not significantly different (n=6 mice per condition, *p<0.05).
accumulation and diffusion of ROS signals to in long-term UCP2 deletion (in utero or postpartum) may allow the partum that could lead to several indirect effects. For example, the mice have UCP2 deleted during embryogenesis and thus UCP2 has been deleted from β-cells during development as well as post-partum that could lead to several indirect effects. For example, the long-term UCP2 deletion (in utero or postpartum) may allow the accumulation and diffusion of ROS signals to influence β-cell function. In contrast, ind.UCP2BKO mice that had UCP2 deleted for a relatively short period of time and CO injected mice showed similar suppression of glucagon levels during the OGTT. Compensatory effects of β-cell UCP2 deletion on α-cell function may not be observed within 4 weeks of deletion and thus the impaired glucose tolerance in the UCP2BKO mice and lack of effect on glucose tolerance in the ind.UCP2BKO mice might be partially explained by differential effects on α-cell function. Future studies will focus on comparing the effect of short-term versus long-term UCP2 deletion on pancreatic β-cell function, ROS production, insulin secretion and whole body glycemia in the ind.UCP2BKO mice. These studies will be very important in determining the exact role of UCP2 in the β-cell and its contribution to the progression of diabetes.

Author Disclosures
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Author Contributions
QG researched the data, contributed to the experimental design and the discussion, and wrote and edited the manuscript. CAR-D researched the data, contributed to the experimental design and the discussion, and edited the manuscript. EMA researched the data, contributed to the experimental design and the discussion, and edited the manuscript. MBW contributed to the experimental design and the discussion, and edited and reviewed the manuscript.

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