Reduced vascular responses to soluble guanylyl cyclase but increased sensitivity to sildenafil in female rats with type 2 diabetes

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First published May 8, 2015; doi:10.1152/ajpheart.00079.2015—Impaired nitric oxide (NO), soluble guanylyl cyclase (sGC), and cyclic guanosine monophosphate (cGMP) signaling (NO-sGC-cGMP) has been implicated in the pathogenesis of diabetic vascular dysfunction. Efforts to directly target this signaling have led to the development of NO agonists that activate the heme group of sGC (stimulators) or preferentially activate sGC when the heme is oxidized (activators). In this study, we hypothesized that resistance arteries from female rats with spontaneous type 2 diabetes (Goto-Kakizaki rats, GK) would have reduced vasodilatory responses to NO-dependent sGC activation and increased responses to NO-independent sGC activation compared with control rats (Wistar). Endothelium-dependent and -independent relaxation was assessed in isolated segments from mesenteric resistance arteries (MA) mounted in a wire myograph. GK MA had reduced responses to acetylcholine (pEC50: 7.96 ± 0.06 vs. 7.66 ± 0.05, P < 0.05) and sodium nitroprusside (pEC50: 8.34 ± 0.05 vs. 7.77 ± 0.04, P < 0.05). There were no group differences in 8-bromoguanosine cGMP-induced relaxation and protein kinase G1 expression (P > 0.05). GK MA had attenuated responses to BAY 41–2272 (heme-dependent sGC stimulator; pEC50: 7.56 ± 0.05 vs. 6.93 ± 0.06, P < 0.05) and BAY 58–2667 (heme-independent sGC activator; pEC50: 10.82 ± 0.07 vs. 10.27 ± 0.08, P < 0.05) and increased sensitivity to sildenafil [phosphodiesterase 5 (PDE5) inhibitor; pEC50: 7.89 ± 0.14 vs. 8.25 ± 0.13, P < 0.05]. Isolated resistance arteries from female rats of reproductive age that spontaneously develop type 2 diabetes have increased sensitivity to PDE5 inhibition and reduced responsiveness to sGC activators and stimulators.

vasorelaxation; type 2 diabetes; vascular smooth muscle; soluble guanylyl cyclase agonists; sildenafil

MACROVASCULAR AND MICROVASCULAR complications are distinctive features of type 2 diabetes and contribute to an increased prevalence of hypertension and risk of cardiovascular disease-associated mortality in diabetic patients (28). Dysfunction of the endothelial layer in the vascular wall contributes to diabetic vascular complications (12, 15); however, emerging evidence suggests that the smooth muscle is also functionally impaired in diabetic vessels and may be a novel therapeutic target of diabetes-associated vascular disease (21, 26).

Diabetic patients and rodent models with experimental diabetes have reduced responsiveness of vascular smooth muscle to nitric oxide (NO) (11, 20, 39, 41), suggesting that the signaling pathway downstream of NO is impaired. Soluble guanylyl cyclase (sGC) is the intracellular receptor of NO (4). Upon its release in endothelial cells, NO reaches the vascular smooth muscle cells by diffusion and binds to sGC, leading to accumulation of intracellular cyclic guanosine monophosphate (cGMP) levels (22). Increased levels of cGMP mainly activate cGMP-dependent protein kinase (PKG) and lead to vasodilation resulting from various events, including reduction of intracellular Ca2+ levels and membrane hyperpolarization (17, 22, 40). To terminate the NO-sGC-cGMP signaling, intracellular cGMP is hydrolyzed by phosphodiesterase 5 (PDE5) (24, 29).

The NO-sGC-cGMP pathway is impaired in diabetes, contributing to reduced endothelium-dependent and -independent relaxation. Various components of this pathway have been used as pharmacological targets to promote vasodilation in disease states (i.e., organic nitrates, NO donors, and PDE5 inhibitors). Most recently, efforts have focused on the development of pharmacological compounds that can directly activate sGC (35). These compounds are divided into two categories, sGC stimulators and activators (23). sGC stimulators enhance the sensitivity of sGC to NO by stabilizing the nitrosyl-heme complex or by increasing sGC activity in the absence of NO (32, 35). The actions of sGC stimulators depend on the presence of a reduced (ferrous) prosthetic heme. Therefore, they are not effective when heme is oxidized or lost, such as in disease states of oxidative stress. On the contrary, sGC activators preferentially activate sGC when the enzyme is in the oxidized or heme-free state (36). Accordingly, selective targeting of oxidized heme with sGC activators has been proposed as an effective pharmacological tool to promote vasodilation in diseased vessels (37). However, the impact of sGC stimulators and activators in vascular dysfunction in type 2 diabetes is unclear.

Most of the studies in the area of diabetes-associated vascular dysfunction have been conducted in older individuals with long-lasting diabetes (37). Studies in experimental diabetes have used animals with extreme and uncontrolled glycemia, which more accurately represent the human condition in its later stage or under no medication. In addition, although it is known that the burden of cardiovascular disease is greater in women with diabetes compared with diabetic men (14), previous studies have primarily included diabetic men or male animal models of diabetes. In the present study, we investigated the function of sGC and its downstream signaling in resistance mesenteric arteries from young female rats with spontaneous type 2 diabetes. The Goto-Kakizaki rat was produced by selective inbreeding of glucose-intolerant Wistar rats (10). Goto-Kakizaki rats present with mild hyperglycemia,
glucose intolerance, vascular dysfunction, and high blood pressure and are not obese, allowing the investigation of molecular mechanisms independent of the confounding effects of obesity (10, 11, 27, 30). In this study, we hypothesized that resistance arteries from Goto-Kakizaki rats would have reduced vasodilatory responses to NO-independent/heme-dependent sGC activation and increased responses to NO- and heme-independent sGC activation.

MATERIALS AND METHODS

Animals and experimental design. Female Goto-Kakizaki rats (in-house bred, derived from the Tampa colony) and age-matched Wistar rats (Charles River Laboratories International, Wilmington, MA) were housed in a temperature- and humidity-controlled environment under 12-h:12-h light/dark cycles and fed with standard laboratory rodent Chow and water. Rats were studied at 20–22 wk of age at the diestrus stage of their estrus cycle determined by microscopic examination of cellular changes in vaginal smears (11). All animal care and experimental procedures were performed in accordance with the Georgia Regents University Committee on the Use of Animals in Research and Education.

Blood glucose and blood pressure measurements. Whole blood glucose was determined from tail blood samples (FreeStyle Lite, Alameda, CA) before vascular reactivity studies. Rats were anesthetized with isoflurane via a nose cone for surgical procedures (initially with 5% and then maintained at 2% in 100% oxygen). A P-10 catheter was inserted into the right femoral artery, and blood pressure was recorded via an attached pressure transducer. After a stable recording of blood pressure for 10 min, rats were killed by removal of their hearts.

Tissue preparation. Following death, the mesenteric arcade was quickly removed and placed in ice-cold physiological salt solution (PSS) of the following composition (in mM): 130 NaCl, 4.7 KCl, 14.9 NaHCO3, 5.5 dextrose, 1.18 KH2PO4, 1.17 MgSO4, 1.6 CaCl2, and 100 mM sodium orthovanadate (Na3VO4). 100 mM PMSF, and 1% protease inhibitor cocktail (Sigma, St. Louis, MO). Homogenates were centrifuged at 10,000 g for 15 min at 4°C, the supernatant was collected, and the proteins were solubilized in Laemmli buffer containing mercaptoethanol. Protein concentration was measured by bicinchoninic acid assay (Thermo Scientific).

Data analysis. Sigmoidal curve fitting was performed on concentration-response data using GraphPad Prism software (v. 6.0; GraphPad Software, San Diego, CA). From this analysis, the maximal effect generated by the agonist (maximum vasodilatation) and the molar concentration of agonist producing 50% of the maximum response (EC50) were determined and presented as Emax and pEC50 (negative logarithm to base 10 of the EC50), respectively. Emax was expressed relative to the maximal changes from the contraction produced by PE in each segment, which was determined as 0% relaxation. The baseline tension before addition of PE was considered 100% relaxation.

Statistical analysis. Values are presented as means ± SE, and n represents the number of animals used in the experiments. Group differences (Wistar vs. Goto-Kakizaki) in Emax, pEC50, and protein expression were determined using Student’s t-tests for unpaired observations. Repeated-measures two-way ANOVA [factor 1: group (Wistar vs. Goto-Kakizaki) vs. drug (drug alone vs. drug plus inhibitor), factor 2: drug concentrations] followed by Bonferroni’s or Tukey’s post hoc tests was used to make comparisons in concentration-response curves. Two-way ANOVA [factor 1: group (Wistar vs. Goto-Kakizaki), factor 2: drug (drug alone vs. drug plus inhibitor)] followed by Bonferroni’s post hoc test was used to determine group differences in pEC50. GraphPad Prism (v. 6.0; GraphPad Software) was used for all statistical analyses. The significance level of all tests was set at α = 0.05.
RESULTS

Increased arterial blood pressure in Goto-Kakizaki rats. Goto-Kakizaki rats weighed less compared with age-matched Wistar controls (246.9 ± 6.0 g vs. 281.8 ± 5.0 g, P < 0.05) and had greater blood glucose levels (133.3 ± 6.8 mg/dl vs. 95.4 ± 2.6 mg/dl, P < 0.05) and higher mean arterial pressure (112 ± 3 mmHg vs. 88 ± 1 mmHg, P < 0.05).

Reduced NO-dependent relaxation in mesenteric arteries from female Goto-Kakizaki rats. Endothelium-intact mesenteric arteries from Goto-Kakizaki rats had reduced relaxation responses to ACh [pEC50: Wistar (n = 13): 7.66 ± 0.05, P < 0.05] and SNP [pEC50: Wistar (n = 12): 8.34 ± 0.05 vs. Goto-Kakizaki (n = 14): 7.77 ± 0.04, P < 0.05] compared with arteries from Wistar rats (Fig. 1, A and B). NOS inhibition reduced relaxation responses to ACh (Fig. 2, A and B), and this effect was smaller in arteries from Goto-Kakizaki compared with Wistar rats [Emax (%relaxation to PE): Wistar (n = 6), vehicle: 96.2 ± 1.8, l-NNA: 46.6 ± 8.0 vs. Goto-Kakizaki (n = 8), vehicle: 93.9 ± 3.3, l-NNA: 71.0 ± 9.2; P < 0.05]. We calculated the magnitude of reduction in ACh-induced relaxation in the presence of l-NNA and used it as an index of NO contribution to ACh-induced relaxation (34). The relative contribution of NO to ACh-induced relaxation was diminished in mesenteric arteries from Goto-Kakizaki rats compared with controls [%Δarea under the curve, Wistar (n = 6): 65.5 ± 4.2 vs. Goto-Kakizaki (n = 8): 46.4 ± 6.4, P < 0.05; Fig. 2C]. sGC inhibition by ODQ abolished relaxation responses to SNP in both groups (data not shown).

Increased expression of sGC but reduced relaxation responses to sGC activation in small mesenteric arteries from Goto-Kakizaki rats. Mesenteric arteries from Goto-Kakizaki rats (n = 5) had increased expression of sGC (both subunits α and β) compared with arteries from Wistar rats (n = 5) (Fig. 3, A and B). BAY 41-2272 induced relaxation responses in a concentration-response manner in arteries from Goto-Kakizaki and Wistar rats, but these responses were reduced in Goto-Kakizaki arteries [pEC50, Wistar (n = 8): 7.56 ± 0.05 vs. Goto-Kakizaki (n = 10): 6.93 ± 0.06, P < 0.05; Fig. 4, A and D]. Treatment with ODQ, an inhibitor of sGC, caused a rightward shift of the concentration-response curve to BAY 41-2272 in both groups (n = 6/group; Fig. 4, B and C) and abolished the group differences (Fig. 4D). BAY 58–2667 induced relaxation responses in arteries from both groups, but these responses were diminished in arteries from Goto-Kakizaki rats [pEC50, Wistar (n = 7): 10.82 ± 0.07 vs. Goto-Kakizaki (n = 9): 10.27 ± 0.06, P < 0.05; Fig. 5, A and D]. ODQ improved mesenteric artery relaxation responses to BAY 58–2667 in Goto-Kakizaki but had no effect on BAY 58-2667-induced relaxation responses in arteries from Wistar rats (n = 6/group; Fig. 5, B–D).

No differences in relaxation responses to cGMP and PKG activation between mesenteric arteries from Goto-Kakizaki and Wistar rats. To assess the integrity of the sGC-cGMP-PKG signaling downstream of sGC, we performed concentration-response curves to a mimetic of cGMP, 8-Br-cGMP, and measured expression of PKG (PKG-1, including both α and β isoforms). 8-Br-cGMP induced relaxation responses in a concentration-response manner, but there were no differences between groups (Wistar, n = 7; Goto-Kakizaki, n = 11; Fig. 6A). Furthermore, there were no group differences in PKG expression (n = 6/group; Fig. 6B).

Increased relaxation responses to PDE5 inhibition in mesenteric arteries from Goto-Kakizaki rats. Sildenafil, a potent and selective inhibitor of PDE5, caused concentration-dependent relaxation in mesenteric arteries (Fig. 7A). Arteries from Goto-Kakizaki rats had increased sensitivity to sildenafil [pEC50, Wistar (n = 6): 7.89 ± 0.14 vs. Goto-Kakizaki (n = 8): 8.25 ± 0.13, P < 0.05] but reduced expression of PDE5 compared with Wistar controls (n = 6/group; Fig. 7B).

DISCUSSION

The main finding of this study was that resistance mesenteric arteries from female rats with type 2 diabetes had reduced responses to NO-dependent and -independent activation of sGC but greater sensitivity to inhibition of PDE5 compared with nondiabetic rats.

There is a strong association between hypertension and diabetes, with hypertension affecting >50% of diabetic patients and contributing to their increased risk for cardiovascular disease (16). Women with type 2 diabetes have greater risk for cardiovascular-related mortality (13), but the mechanisms un-
underlying this risk are not clear. In this study, we used the Goto-Kakizaki rat, a genetic model of type 2 diabetes with mild hyperglycemia, elevated blood pressure, and vascular dysfunction (11, 30). Resistance mesenteric arteries from young female Goto-Kakizaki rats had reduced responses to ACh, an indication of endothelial dysfunction. Our results are in agreement with previously published data in patients and other animal models with diabetes (15, 38) and support the overall consensus that endothelial dysfunction is a hallmark of diabetic vasculopathy. In previous studies, male Goto-Kakizaki rats of the same age but with greater blood glucose levels did not show reduced mesenteric artery responses to ACh unless they were fed with a high-fat diet (30), indicating potential sex differences in the vascular phenotype of this animal model.

Recently, we reported that, in addition to endothelial dysfunction, uterine arteries from Goto-Kakizaki rats had impaired vascular smooth muscle relaxation responses (11). In the present study, resistance arteries from a nonreproductive vascular bed showed a similar defect, suggesting that dysfunction of the smooth muscle layer is a feature of vascular dysfunction in the Goto-Kakizaki animal model of type 2 diabetes. This finding and our previous studies are in support of earlier reports in conduit arteries from male Goto-Kakizaki rats (42) and studies in humans showing reduced endothelium-independent relaxation in patients with type 2 diabetes (20, 39, 41).

The protein expression of sGC, the intracellular receptor of NO, was increased (both a1 and a2 subunits) in resistance vessels of Goto-Kakizaki rats. Furthermore, the contribution of NO to endothelium-dependent relaxation was attenuated in resistance arteries from diabetic rats. Thus the increased sGC expression may be a compensatory mechanism to counteract the reduced vasodilatory stimulus. In previous studies, basal levels of sGC protein did not differ between Goto-Kakizaki and Wistar rats in uterine arteries (11) and aortae (42), suggesting that the adaptations of the NO-sGC-cGMP pathway are vascular bed specific. These adaptations may also be modulated by sex and age, as the studies in aortae of Goto-Kakizaki rats were conducted in older male animals (42). The use of a cGMP analog elicited similar relaxation responses in Wistar and Goto-Kakizaki rats. In addition, the protein levels of PKG

Fig. 2. Nitric oxide (NO) contribution to ACh-induced relaxation in mesenteric arteries from female Wistar and GK rats. Inhibition of NO synthase with NOS-nitro-l-arginine (l-NNa) (10^-4 M) reduced relaxation responses to ACh in mesenteric arteries from Wistar (n = 6) (A) and GK (n = 8) rats (B) (aP < 0.05, ACh plus vehicle vs. ACh plus l-NNa). PE, phenylephrine. C: relative contribution of NO to ACh-induced relaxation was reduced in mesenteric arteries from GK rats [P < 0.05, Wistar (n = 6) vs. GK (n = 8)]. AUC, area under the curve.

Fig. 3. Protein expression levels of soluble guanylyl cyclase-a (sGCa) (A) and sGCb (B) in mesenteric resistance arteries from female Wistar and GK rats. Protein levels of sGCa and sGCb were increased in mesenteric resistance arteries from GK (n = 5) compared with Wistar (n = 5) rats. Values are means ± SE. *P < 0.05, Wistar vs. GK.
did not differ in mesenteric arteries from diabetic rats compared with control animals. Collectively, these data suggest that the reduced relaxation responses seen in the present study are attributed to defects at the level of sGC and beyond, including reduced production/bioavailability of NO and reduced sensitivity of sGC.

We used various agonists to activate sGC and induce smooth muscle relaxation and found that the arteries from the diabetic...
group had reduced dilatory responses regardless of the agonist used (i.e., NO donor vs. heme-dependent vs. heme-independent sGC activation). On the basis of previous findings demonstrating that sGC activators (i.e., BAY 58–2667) preferentially activate sGC when the enzyme is in the oxidized state (36) and evidence showing that vascular damage in diabetes is often due to hyperglycemia-induced generation of reactive oxygen species (5), we had initially speculated that mesenteric arteries from female diabetic rats would have reduced vascular relaxation responses to a sGC stimulator but enhanced responses to a sGC activator. This hypothesis was further supported by previous reports demonstrating that male rats with type 2 diabetes had preserved responses to the heme-independent activator protoporphyrin-IX (42) and that isolated vessels from patients with type 2 diabetes had enhanced relaxation responses to BAY 58–2667 (37). In contrast to our hypothesis and these previous findings, we found reduced responses to BAY 58–2667 in female Goto-Kakizaki compared with Wistar rats. It is noteworthy, however, that the study by Witte et al. (42) examined the function of sGC in aorta, a conduit artery, compared with our investigation in mesenteric resistance arteries, and they used older male animals. Furthermore, studies in isolated vessels from diabetic patients were performed in older (50–75 yr) adults, and their sex and medication regimens, which could be potential confounding factors, were not reported (37).

Vascular reactivity studies in animal models of diabetes have revealed contradictory results with variable responses to vasoconstrictors and vasodilators (1). The duration of diabetes plays an important role in this variability because previous reports showed enhanced responses to ACh 1 day after the induction of diabetes, unchanged responses at 1–2 wk, and reduced responses at 8 wk after diabetes induction (25). Similar trends were seen in vasoconstrictor responses (43). Time-dependent alterations of endothelial function have been previously studied in animal models of diabetes (1, 25), but the temporal changes in vascular oxidative stress and smooth muscle dilatory responses have not been extensively investi-

![Fig. 6. Concentration-response curves to a cyclic guanosine monophosphate (cGMP) mimetic and protein levels of protein kinase G (PKG) in mesenteric arteries from female Wistar and GK rats. A: there were no differences between mesenteric arteries from GK (n = 7) and Wistar rats (n = 11) in 8-bromo-guanosine cGMP (8-Br-cGMP) concentration-response curves (P > 0.05, Wistar vs. GK). B: protein levels of PKG were not different between Wistar and GK mesenteric arteries (P > 0.05, Wistar vs. GK; n = 6/group).](http://ajpheart.physiology.org/)

![Fig. 7. Concentration-response curves to sildenafil [a phosphodiesterase 5 (PDE5) inhibitor] and protein levels of PDE5A in mesenteric resistance arteries from female Wistar and GK rats. A: mesenteric arteries from GK rats (n = 8) had greater relaxation responses to sildenafil compared with arteries from Wistar rats (n = 6). Inset: group comparisons for sildenafil pEC50. (*P < 0.05, Wistar vs. GK). B: protein levels of PDE5 were lower in mesenteric arteries from GK rats (*P < 0.05, Wistar vs. GK; n = 6/group).](http://ajpheart.physiology.org/)
gated. Interestingly, the presence of ODQ, which inhibits sGC by oxidation of the ferrous iron in the heme cofactor (7), potentiated the responses to BAY 58–2667 in diabetic but not in control arteries. These data suggest that vascular sGC from female Goto-Kakizaki rats may be more susceptible to oxidation. Therefore, in the presence of the same amount of ODQ, the levels of heme oxidation may be greater in the diabetic rats, favoring greater responses to BAY 58–2667. An alternative interpretation of these results is that ODQ potentiates the vasodilatory effects of BAY 58–2667, and thus a pharmacological approach combining the two compounds may promote better dilatory responses in arteries from animals with mild hyperglycemia.

Resistance mesenteric arteries from Goto-Kakizaki rats had reduced responses to sGC agonists but increased sensitivity to sildenafil, a PDE5 inhibitor. PDE5 is a metallo hydrolase that catalyzes the breakdown of cGMP into the inactive 5'-GMP (18). The binding of cGMP to GAF allostERIC binding site in PDE5 promotes PKG-mediated phosphorylation and activation of the enzyme (6, 19). PDE5 is highly expressed in vascular cells, and its physiological importance in regulating vascular tone has been highlighted by the use of its specific inhibitors (i.e., sildenafil), which promote vasodilation (18). Currently, PDE inhibitors are approved by the US Food and Drug Administration for the treatment of erectile dysfunction, pulmonary hypertension, and benign prostatic hyperplasia. Studies support the beneficial effects of sildenafil in diabetes, as they have shown that chronic treatment with PDE inhibitors, including sildenafil, improves cardiomyopathy in diabetic patients (9). A recent meta-analysis, however, pointed out the lack of data in sex differences in the efficacy of sildenafil in cardiovascular disease (8). Denardo et al. (3) reported that PDE5 inhibition acutely improves microvascular dysfunction in women with symptoms and signs of cardiac ischemia. On the other hand, studies in female mice have shown that the cardioprotective effects of chronic treatment with sildenafil were dependent on the presence of estrogen, as sildenafil failed to induce antiremodeling effects after ovariectomy, whereas estrogen replacement restored the beneficial effects of sildenafil (31). Others have demonstrated that estrogen withdrawal is a risk factor for flushing induced by PDE5 inhibition (33). Of note, we have previously reported that estradiol levels are greater in Goto–Kakizaki rats compared with Wistar rats (2). Together, this evidence and our results suggest that PDE5 inhibition may have beneficial effects on cardiovascular function of women with type 2 diabetes during their reproductive age, when estrogen is at normal levels. Whether hormonal variations in premenopausal diabetic women contribute to greater sensitivity to PDE5 inhibition is unknown.

In this study, we used lean rats with type 2 diabetes. This approach increased the internal validity of our experimental design because it allowed the investigation of vasodilatory mechanisms in the absence of the confounding effects of obesity. In the human population, however, type 2 diabetes is often accompanied by an obese phenotype. Thus future studies should examine the contribution of increased adiposity vs. hyperglycemia and insulin resistance on the vascular phenotype seen in the present study. Another limitation of our study is the lack of direct evidence of reduced ability of the diabetic vessels to produce cGMP. Because of the small size of resistance mesenteric vessels, we were unable to measure production of cGMP in response to sGC activation. Previous reports, however, showed reduced levels of cGMP in response to sGC activation in aortae of female GK rats.

The effects of sGC agonists have been primarily studied in males with established diabetes of several years. The recommendation for clinical use of sGC activators and stimulators also relies on studies primarily conducted in men with diabetes or male animal models of diabetes. The rats used in the present study were young, female, and had mild hyperglycemia, representing a female patient at the initial stages of diabetes. The effects of sex on the efficacy of sGC agonists should be addressed before they are used in clinical practice. Previous studies in experimental diabetes have shown that the vascular adaptations to diabetes differ at the early compared with established stages (1, 25). Thus the duration of diabetes and other factors such as levels of glycemia and status of oxidative stress should also be considered. Here, we report, for first time, increased sensitivity of diabetic mesenteric arteries to sildenafil, a PDE5 inhibitor. Similar results have been previously reported in uterine arteries from diabetic rats (11). Future studies should address the hypothesis that PDE5 inhibition may be preferable over sGC agonists in female subjects at the beginning stages of type 2 diabetes.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

Author contributions: S.G., J.L.H., T.M., and R.C.W. conception and design of research; S.G., J.L.H., and S.O. performed experiments; S.G. and T.M. analyzed data; S.G., J.L.H., T.M., A.E., and R.C.W. wrote the paper; S.G., J.L.H., T.M., S.O., and R.C.W. approved final version of manuscript.

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