Central losartan attenuates increases in arterial pressure and expression of FosB/ΔFosB along the autonomic axis associated with chronic intermittent hypoxia

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Knight WD, Saxena A, Shell B, Nedungadi TP, Mifflin SW, Cunningham JT. Central losartan attenuates increases in arterial pressure and expression of FosB/ΔFosB along the autonomic axis associated with chronic intermittent hypoxia. Am J Physiol Regul Integr Comp Physiol 305: R1051–R1058, 2013.—Chronic intermittent hypoxia (CIH) increases mean arterial pressure (MAP) and FosB/ΔFosB staining in central autonomic nuclei. To test the role of the brain renin-angiotensin system (RAS) in CIH hypertension, rats were implanted with intracerebroventricular (icv) cannulae delivering losartan (1 μg/h) or vehicle (VEH) via miniosmotic pumps and telemetry devices for arterial pressure recording. A third group was given the same dose of losartan subcutaneously (sc). Two groups of losartan-treated rats served as normoxic controls. Rats were exposed to CIH or normoxia for 7 days and then euthanized for immunohistochemistry. Intracerebroventricular losartan attenuated CIH-induced increases in arterial pressure during CIH exposure (0800-1600 during the light phase) on days 1, 6, and 7 and each day during the normoxic dark phase. FosB/ΔFosB staining in the organum vasculosum of the lamina terminalis (OVLT), median preoptic nucleus (MnPO), peri-ventricular nucleus of the hypothalamus (PVN), the rostral ventrolateral medulla (RVLM), and the nucleus of the solitary tract (NTS) was decreased in icv losartan-treated rats. Subcutaneous losartan also reduced CIH hypertension during the last 2 days of CIH and produced bradycardia prior to the effect on blood pressure. Following sc losartan, FosB/ΔFosB staining was reduced only in the OVLT, MnPO, PVN, and NTS. These data indicate that the central and peripheral RAS contribute to CIH-induced hypertension and transcriptional activation of autonomic nuclei and that the contribution of the central RAS is greater during the normoxic dark phase of CIH hypertension.

sleep apnea; angiotensin; blood pressure; hypertension

CHRONIC INTERMITTENT HYPOXIA (CIH), an animal model of the arterial hypoxemia that occurs in sleep apnea patients, is associated with increased arterial pressure and sympathetic tone (17, 51). Both the renin-angiotensin system (RAS) (15, 18) and peripheral arterial chemoreceptors (16, 25) contribute to the hypertension associated with CIH. Previous experiments have demonstrated that the contribution of the RAS to CIH-associated hypertension involves both peripheral and central nervous system (CNS) mechanisms (11, 15, 18, 29). In several of these studies, the angiotensin receptor blocker losartan was delivered by gavage or in drinking water (15, 18, 29). It has been demonstrated that peripherally administered losartan can cross the blood-brain barrier to block central angiotensin receptors (26, 35, 38, 52). Thus, some of the antihypertensive effects of peripheral losartan administration in previous studies of CIH may have been due to its central actions. Peripheral administration of losartan has been shown to attenuate chemoreceptor sensitization associated with CIH (29), and chronic angiotensin receptor blockade in the paraventricular nucleus (PVN) of the hypothalamus prevents increases in blood pressure induced by eucapnic intermittent hypoxia (11).

In addition to the PVN, endogenous angiotensin could be active in several other CNS regions that regulate autonomic function during CIH. Chronic transcriptional activation, as measured by FosB immunohistochemistry, is observed in the PVN and also the lamina terminalis, the nucleus of the solitary tract (NTS), and rostral ventrolateral medulla (RVLM) following CIH (23). Angiotensin signaling in the NTS and RVLM and their contributions to central autonomic regulation and hypertension have been well characterized (4, 7, 12, 14, 22, 33, 45). The lamina terminalis contains two circumventricular organs, the subfornical organ (SFO) and the organum vasculosum of the lamina terminalis (OVLT), and the median preoptic nucleus (MnPO) that is located between the SFO and OVLT by 10.220.32.246 on July 3, 2017 http://ajpregu.physiology.org/ Downloaded from

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hypothesis was tested by using continuous intracerebroventricular (icv) and subcutaneous infusions of the angiotensin type 1 receptor blocker losartan during a 7-day CIH protocol.

METHODS

Animals. Adult male Sprague-Dawley rats (250–300 g; Charles River Laboratories, Wilmington, MA) were individually housed and maintained on a 14:10-h light-dark cycle and provided with ad libitum access to food and water. All protocols using animals were conducted according to National Institutes of Health guidelines and approved by the Institutional Animal Care and Use Committee at the University of North Texas Health Science Center in Fort Worth, TX.

Radio telemetry transmitter instrumentation. All rats were anesthetized with isoflurane (2–3%) and implanted with an abdominal aortic catheter attached to a TA11PA-C40 radiotelemetry transmitter (Data Sciences, St. Paul, MN) that was sutured to the abdominal wall, as described previously (23). Rats were allowed to recover for ≥1 wk prior to receiving osmotic minipumps.

Osmotic minipump implantation. All rats were anesthetized with isoflurane (2–3%) for the duration of the surgical procedures. Each rat that received an icv cannula was placed in a stereotaxic frame equipped to deliver isoflurane (Kopf Instruments, Tujunga, CA). A midline incision was made through the disinfected scalp and the skull exposed around bregma. A 22-gauge stainless-steel cannula was implanted into the left lateral ventricle (stereotaxic coordinates: −0.9 mm posterior, 1.4 mm lateral, and −3.9 mm ventral to bregma), as described previously (44). Each cannula was attached via polyethylene tubing to an osmotic minipump (model 2004, 0.25 μL/h; Alzet, Cupertino, CA) delivering 0.9% saline [vehicle (VEH)] or losartan (LOS) at a rate of 1 μg/h. This dose of LOS was selected on the basis of previous studies, indicating that it does not affect baseline blood pressure (39, 49, 50, 53). Cannulae were anchored to the skull using dental acrylic and jeweler’s screws. The osmotic minipump was placed in a subcutaneous pocket made between the scapulae. A third group was instrumented only with a subcutaneous (sc) minipump filled with the same concentration of LOS to control for possible leakage of the drug into the periphery. Rats were allowed to recover from surgery for 5 days prior to baseline recordings.

Chronic intermittent hypoxia protocol. All rats were placed into custom-built intermittent hypoxia chambers 1 wk before the treatment period was begun. Rats were allowed to acclimate to the chambers at normoxia (21% O2) for several days prior to baseline cardiovascular data being recorded for 5 days. Thereafter, all rats were exposed to CIH cycling between 21 and 10% O2 at 8 h/light phase for 7 days according to previously described protocols (23). Normoxic controls receiving icv or sc LOS were housed under similar conditions but were not exposed to hypoxia.

Telemetry monitoring. Mean arterial blood pressure (MAP), heart rate (HR), and respiratory rate (RR) were recorded continuously using a Dataquest IV radio telemetry system (Data Sciences). Data were collected during 10-s sampling periods (500 Hz) that were averaged and recorded every 10 min, as described previously (9). Because of the automated nature of the sampling protocol, data collected during intermittent hypoxia exposure (0800-1600) included periods for nitrogen and room air exposure.

Immunohistochemistry. On day 8, after 16–20 h of the last CIH exposure, rats were anesthetized with thiobutabarbital (100 mg/kg ip; Sigma Chemical, St. Louis, MO) and perfused transcardially with PBS followed by 4% paraformaldehyde, as described previously (23). Each brain was placed in PBS with 30% sucrose for 2–3 days, and each forebrain and brain stem were sectioned separately at 40 μm. Three separate sets of serial sections were collected from each brain and stored in cryoprotectant at −20°C prior to staining (48). One set of serial sections from each rat was processed for FosB (1:1,000, goat anti-FosB; Santa Cruz Biotechnology, Santa Cruz, CA) immunohistochemistry, as described previously (23). Sections were then processed with a biotinylated horse anti-goat IgG (1:200; Vector Laboratories, Burlingame, CA) and reacted with an avidin-peroxidase conjugate (Vectastain ABC Kit; Vector Laboratories) and PBS containing 0.04% 3,3′-diaminobenzidine hydrochloride and 0.04% nickel ammonium sulfate for 10–11 min. Because of the primary antibody used in this study does not discriminate full-length FosB from the splice variant ΔFosB, the staining will be referred to as FosB/ΔFosB.

Tissue sections were analyzed using an Olympus microscope (BX41) equipped for epifluorescence and an Olympus DP70 digital camera with DP manager software (version 2.2.1). Images were converted to 16-bit format and uniformly adjusted for brightness and contrast using Image J (version 1.44p; National Institutes of Health). Regions were identified using a rat brain stereotaxic atlas (34). The anterior border of the RVLM was defined as the caudal pole of the facial nucleus, and the rostral hypoglossal nucleus was used to define the posterior border (−13.80 to −13.30 mm from Bregma), as described previously (10). For the NTS, the analysis included sections containing the subpostremal and commissural subregions of the nucleus (−14.60 to −13.68 mm from Bregma) that contain the main termination sites for chemoreceptor and baroreceptor afferents (40). The rostral portion of the NTS that was for the analysis began at the rostral pole of the area postrema and only included sections in which the NTS extended into the forth ventricle (13.3 to −13.24 mm from bregma). Analysis of FosB/ΔFosB-positive cells in PVN was conducted as described previously (43). The numbers of FosB/ΔFosB-positive cells in each major subregion, dorsal, medial, and lateral parvocellular and lateral magnocellular, were averaged across the rostrocaudal extent of the nucleus and analyzed separately. FosB/ΔFosB-positive cells in each region, identified by the shape and size of the profile as nuclear staining, were counted using Image J. Four to six sections were analyzed from each rat for each region, except for the areas of the lamina terminalis due to their limited rostral-caudal dimensions. For technical reasons, the SFO was not included in the analysis.

Statistics. Effects of CIH on MAP, HR, and RR during the dark phase and during the 8-h period of CIH exposure were analyzed separately by two-way repeated-measures ANOVA (time × treatment). Student-Newman-Keuls (SNK) post hoc tests were used for followup analysis of significant interactions or main effects. Cell counts were analyzed by one-way ANOVA with SNK tests for post hoc analysis. Significance was set at P < 0.05 for all tests. All data are presented as means ± SE.

RESULTS

LOS infusion and cardiorespiratory function. Intracerebroventricular or sc infusions of LOS did not significantly affect MAP or HR prior to the start of CIH (Table 1). Statistical analyses of the changes in MAP and HR during CIH exposure from 0800 to 1600 by two-way repeated-measures mixed-effects ANOVA indicated significant group-by-day interactions [MAP: F(24, 264) = 1.68, P < 0.02; HR: F(24, 264) = 1.67, P < 0.03]. On the first day, both VEH and SC LOS-treated rats exposed to CIH demonstrated significant increases in MAP compared with the other groups (Fig. 1A). For days 2–5 and 7, CIH was associated with a significant increase in MAP versus the normoxic control groups independent of drug treatment. However, on days 6 and 7 of the protocol, the changes in MAP observed in both icv and sc LOS-treated rats exposed to CIH were significantly decreased compared with the icv VEH and CIH group. There were no differences in HR changes between VEH- and icv LOS-treated rats during CIH, whereas sc LOS was associated with a significant bradycardia that was significantly different from the two other groups exposed to CIH beginning on day 3 of the protocol (Fig. 1B).
The changes in HR in the sc LOS and CIH group were not different from the two LOS-treated normoxic control groups. Changes in RR during CIH were not affected by icv or sc LOS, although sc LOS-treated rats demonstrated a trend for smaller changes in RR than the other groups [RR group: F(2, 34) = 2.14, P > 0.05; group X: day: F(14, 238) = 1.03, P > 0.05; data not shown].

Analysis of the changes in MAP and HR during the normoxic dark phase of the CIH protocol also revealed significant group-by-day interactions [MAP: F(24, 264) = 2.46, P < 0.001; HR: F(24, 264) = 1.92, P < 0.01]. Intracerebroventricular LOS significantly attenuated the sustained increase in MAP during the normoxic dark phase for each day of CIH compared with the VEH + CIH group (Fig. 1C). Rats treated with SC LOS demonstrated increases in MAP that were comparable with the VEH and significantly greater than icv LOS rats exposed to CIH as well as the two normoxic control groups during the first 5 days of the CIH protocol (Fig. 1C). On the last 2 days of the protocol, the changes in MAP of the sc LOS and CIH group decreased significantly to levels comparable with normoxic controls. Rats treated with sc LOS also demonstrated a significant bradycardia during the dark phase that was more pronounced during the last 3 days of CIH (Fig. 1D).

LOS infusion and FosB/ΔFosB staining. The numbers of FosB/ΔFosB-positive cells in the OVLT and MnPO of rats treated with icv VEH and CIH were significantly greater than all of the other treatment groups (Fig. 2). Subcutaneous LOS decreased these CIH effects significantly, but icv LOS produced a significantly greater attenuation in both regions. However, FosB/ΔFosB staining in the OVLT of the icv LOS group was still significantly increased compared with the two normoxic control groups, which demonstrated little to no FosB/ΔFosB immunoreactivity. This was not the case in MnPO, where the numbers of FosB/ΔFosB-positive cells in the icv LOS and CIH rats were not different from the two LOS-treated normoxic control groups.

Similar results were observed in the dorsal and medial parvocellular subregions of the PVN. The numbers of FosB/ΔFosB-positive cells in the dorsal, medial, and lateral parvocellular regions of the PVN of CIH in VEH-infused rats were significantly greater than all other groups (Fig. 2). In each of these regions of parvocellular PVN, FosB/ΔFosB staining in icv LOS- and CIH-treated rats was significantly reduced to levels that were not different from the normoxic control groups. In the sc LOS and CIH group, FosB/ΔFosB staining in dorsal and medial parvocellular PVN was significantly reduced.

**Table 1.** Baseline averages for MAP, HR, and RR recorded during 0800-1600 (CIH) and the entire DK for rats treated with icv VEH, icv LOS, and sc LOS

<table>
<thead>
<tr>
<th>Group (Time)</th>
<th>MAP, mmHg</th>
<th>HR, beats/min</th>
</tr>
</thead>
<tbody>
<tr>
<td>VEH (n = 12)</td>
<td>94.8 ± 2</td>
<td>322 ± 5</td>
</tr>
<tr>
<td>CIH</td>
<td>99.6 ± 2</td>
<td>379 ± 6</td>
</tr>
<tr>
<td>sc LOS (n = 17)</td>
<td>96.0 ± 1</td>
<td>337 ± 6</td>
</tr>
<tr>
<td>CIH</td>
<td>100.5 ± 1</td>
<td>387 ± 6</td>
</tr>
</tbody>
</table>

Values are means ± SE. MAP, mean arterial pressure; HR, heart rate; RR, respiratory rate; CIH, chronic intermittent hypoxia; DK, dark period; icv, intracerebroventricular; VEH, vehicle; LOS, losartan; sc, subcutaneous. There were no statistically significant differences among the treatment groups (2-way repeated-measures ANOVA).
compared with the icv VEH and CIH group but was significantly greater than the three other treatment groups. In lateral parvocellular PVN, sc LOS and CIH treatment was not different from icv VEH and CIH. In magnocellular PVN, there were low levels of FosB/FosB staining that were not different among the groups. Representative photomicrographs of FosB/FosB staining for each region and treatment group are shown in Figs. 3 and 4.

In the brain stem, FosB/FosB staining observed in the RVLM of the icv VEH and CIH and sc LOS and CIH groups was significantly increased compared with the other three groups (Fig. 5). The numbers of FosB/FosB-positive cells in the commissural and subpostremal regions of the NTS were significantly increased in the icv VEH- and CIH-treated rats compared with all other groups (Fig. 5). In both of the regions of the NTS, icv and sc LOS significantly reduced FosB/FosB staining associated with CIH by comparable amounts that were still significantly greater than the normoxic control groups. Neither icv LOS nor sc LOS significantly affected CIH-induced FosB/FosB staining in more rostral sections of the NTS. Representative photomicrographs of FosB/FosB staining in the commissural NTS and RVLM are shown in Fig. 6.

**DISCUSSION**

Intracerebroventricular LOS infusions significantly and consistently attenuated the sustained component of CIH hypertension during the normoxic dark phase. In addition, icv LOS also attenuated CIH-induced increases in MAP during both CIH exposure (0800-1600) but only during the first day and the last
2 days of the protocol. Similar changes in MAP were not observed in icv LOS-treated rats maintained in normoxia during either part of the light-dark cycle. Intracerebroventricular LOS did not affect either RR or HR during any part of the protocol. These results could suggest that the central RAS participates in the increases in blood pressure that occur in both the sustained component observed during the normoxic dark phase and while the rats are exposed to hypoxia, although icv LOS had much more consistent effects on the sustained component of the hypertension during normoxia. Furthermore, FosB/FosB staining associated with CIH in each of the brain regions examined was significantly reduced in icv LOS-treated rats. These results suggest that central angiotensin receptor blockade decreases transcriptional activation in key brain regions involved in autonomic and endocrine control of blood pressure. The reduction in FosB/FosB staining could represent a direct effect of LOS in each area or be a consequence of reduced activity in other regions. It is also possible that the consistent reductions in MAP that occurred during the normoxic dark phase contributed to the decreases in FosB/FosB staining in baroreceptor sensitive regions such as the NTS.

Although sc LOS infusions were used as a control for possible peripheral effects of icv LOS, it produced a number of distinct effects that were not evident in icv-treated rats. First, sc LOS infusions reduced significantly the CIH-mediated increases in MAP only during the last 2 days of the protocol, whereas icv LOS’s effects were observed earlier and more consistently during CIH and the normoxic dark phase. In addition, sc LOS attenuated the increases in HR during CIH and produced bradycardia during the normoxic dark phase that were evident prior to the effects of sc LOS on blood pressure. These changes in MAP and HR were not observed in the normoxic control given sc LOS. FosB/FosB staining was significantly decreased by sc LOS infusion in the OVLT, MnPO, and the dorsal and medial parvocellular regions of the PVN compared with the vehicle-infused rats exposed to CIH. However, in each of these regions, FosB/FosB staining in the sc LOS-treated rats exposed to CIH was significantly increased compared with the icv LOS and CIH group as well as normoxic controls treated with LOS. Intracerebroventricular and sc LOS infusions did not affect

Fig. 4. Representative digital images of FosB/FosB staining in the medial paraventricular nucleus in rats treated with VEH & CIH (A), LOS icv & CIH (B), and LOS sc & CIH (C), icv LOS & normoxia (D), and sc LOS and Norm (E). Each image was adjusted for uniform brightness and contrast. Scale bar, 100 μm.

Fig. 5. Mean no. of FosB/FosB-positive cells in rostral ventrolateral medulla (RVLM) and the commissural (cNTS), subpostremal (spNTS), and rostral (rNTS) regions of the nucleus of the solitary tract in VEH & CIH-, icv LOS & CIH-, sc LOS & CIH-, icv LOS & Norm-, and sc LOS & Norm-treated rats.

*P < 0.05 compared with all other groups; +P < 0.05 from other groups but not from groups with the same symbol (n = 6–11).
baseline MAP or HR prior to CIH, as reported previously (39, 49, 50, 53), suggesting that these doses of LOS did not interfere with the maintenance of basal MAP or HR.

Given the differences in the effects of icv and sc LOS on CIH-induced responses, it seems unlikely that the effects observed during icv LOS were due to systemic diffusion of losartan. If this were the case, decreases in HR during both CIH and the normoxic dark phase should have been associated with icv infusion, and the effects of icv LOS on MAP might have occurred later in the protocol instead of on the first day of CIH. Furthermore, the effects of sc LOS on CIH could be due to peripheral and not central actions of the antagonist. For example, angiotensin has been shown to influence baroreceptor regulation of HR through AT1 receptors by shifting the reflex to higher operating pressures (6, 45), an effect also observed in some studies on CIH (24, 27, 41). The bradycardia produced

Fig. 6. Representative digital images of FosB/ΔFosB staining in the cNTS (left) and RLVM (right) of rats exposed to VEH & CIH (A), LOS icv & CIH (B), and LOS sc & CIH (C), icv LOS & Norm (D), and sc LOS & Norm (E). Each image was adjusted for uniform brightness and contrast. Scale bar, 100 μm.
by sc LOS could be a result of peripherally acting LOS blocking a possible angiotensin-mediated shift in baroreflex function. Similarly, the effect of sc LOS on CIH-induced hypertension could be related to peripheral angiotensin’s demonstrated role in endothelial dysfunction (30) and chemoreceptors (29). The carotid body has been shown to contain AT1 receptors, and angiotensin was demonstrated to produce dose-dependent increases in sinus nerve activity in vitro (1). Moreover, peripheral angiotensin also has direct excitatory effects on sympathetic ganglia (3, 13, 28) that could have been affected by sc LOS. Whichever of these effects is responsible alone or in combination with the reduction in MAP associated with sc LOS infusion, they do not appear to be necessary for the induction of CIH-induced hypertension since MAP was not affected by sc LOS until the last 2 days of CIH exposure. This contrasts with the effects of the icv LOS infusions that affected CIH-induced hypertension during CIH-induced and in the sustained component of the hypertensive response during the normoxic dark phase starting on the first day of exposure and consistently attenuated the sustained dark phase component of the hypertensive response throughout the 7-day protocol. It is also possible that the sc LOS infusion effects were delayed because the infusion did not produce adequate AT1 receptor blockade until the last 2 days of the CIH protocol, which would have been 16 and 17 days after implantation of the minipumps.

Based on the results of the FosB/ΔFosB data, another possible mechanism could be proposed to account for the late reduction in CIH hypertension produced by sc LOS. Subcutaneous LOS selectively reduced FosB/ΔFosB staining in the OVLT, a forebrain circumventricular organ, and in the NTS, which is the primary target of chemosensory afferents. These results could represent reduced transcriptional activation in these two areas resulting from the inhibition of AT1 receptors accessible to blood-borne LOS in the OVLT (2, 31) and on chemoreceptors projecting to the NTS (1). These effects could have reduced centrally mediated sympathetic tone, although this effect may not have been reflected in changes in FosB/ΔFosB staining of regions inside the blood-brain barrier due to the long half-life of FosB/ΔFosB (21, 36).

The effects of icv LOS on CIH-induced hypertension were evident in both the increases in MAP observed during CIH (0800-1600) and the normoxic dark phase, although they were more consistent during the latter. This contrasts with our previous study, which showed that electrolytic lesions of the lamina terminalis and dominant negative inhibition of FosB in the MnPO selectively reduced hypertension during the dark phase but not during CIH exposure (9). The differences in these results could be due to the effects of LOS in other regions such as the PVN, RVLM, and NTS in addition to blocking AT1 receptors associated with the lamina terminalis. For example, a previous study demonstrated that the inhibition of AT1 receptors in the PVN reduces CIH hypertension (11). This speculation is also supported by the observation that icv LOS reduced FosB/ΔFosB staining in the NTS, a result that was not observed following dominant negative inhibition of MnPO ΔFosB. Intracerebroventricular LOS acting in the NTS could have reduced transcriptional activation in this region, contributing to the significant decrease in MAP that occurred when the rats were exposed to CIH. Additional experiments will be needed to determine how angiotensin receptors and FosB/ΔFosB in each of these regions contribute to CIH hypertension.

**Perspectives and Significance**

Fletcher and colleagues (15, 18) described a link between the traditional systemic RAS and CIH-induced hypertension when this model was initially characterized. Our results support this body of work and provide additional evidence that the brain RAS contributes to CIH hypertension. Furthermore, the brain RAS appears to be critical for the development and maintenance of the sustained hypertension during normoxia. During intermittent hypoxia (0800-1600), this system appears to be less critical because the observed increase in blood pressure was not different from VEH-treated rats during the middle 3 days of the CIH protocol. However, icv LOS delayed the onset and later attenuated the blood pressure responses during CIH exposure, indicating a role for central AT1 receptors in the regulation of blood pressure during this component of the protocol. These results and that of our previous study (9) suggest that different CNS mechanisms may be responsible for the development and maintenance of CIH-induced hypertension during intermittent hypoxia exposure versus normoxia. Additional research addressing the contribution of the brain regions identified in this study will be necessary to determine the scope of their involvement of the brain RAS in CIH-induced hypertension. The network and cellular mechanisms contributed by the brain RAS in CIH hypertension could be relevant to other experimental models of hypertension or other types of clinical hypertension.

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**GRANTS**

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**DISCLOSURES**

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

**AUTHOR CONTRIBUTIONS**

W.D.K., B.S., and T.P.N. performed the experiments; W.D.K., A.S., B.S., T.P.N., S.W.M., and J.T.C. analyzed the data; W.D.K., A.S., B.S., T.P.N., S.W.M., and J.T.C. edited and revised the manuscript; J.T.C. contributed to the conception and design of the research; J.T.C. approved the final version of the manuscript.

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