Sympathetic activation increases NO release from eNOS but neither eNOS nor nNOS play an essential role in exercise hyperemia in the human forearm

Husain Shabeeh,1 Michael Seddon,1 Sally Brett,1 Narbeh Melikian,1 Barbara Casadei,2 Ajay M. Shah,1 and Phil Chowienczyk1

1King’s College London British Heart Foundation Centre, London, United Kingdom; and 2Department of Cardiovascular Medicine, University of Oxford, Oxford, United Kingdom

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Shabeeh H, Seddon M, Brett S, Melikian N, Casadei B, Shah AM, Chowienczyk P. Sympathetic activation increases NO release from eNOS but neither eNOS nor nNOS play an essential role in exercise hyperemia in the human forearm. Am J Physiol Heart Circ Physiol 304: H1225–H1230, 2013. First published February 22, 2013; doi:10.1152/ajpheart.00783.2012.—Nitric oxide (NO) release from endothelial NO synthase (eNOS) and/or neuronal NO synthase (nNOS) could be modulated by sympathetic nerve activity and contribute to increased blood flow after exercise. We examined the effects of brachial-arterial infusion of the nNOS selective inhibitor S-methyl-l-thiocitrulline (SMTC) and the nonselective NOS inhibitor Nω-monomethyl-L-arginine (L-NMMA) on forearm arm blood flow at rest, during sympathetic activation by lower body negative pressure, and during lower body negative pressure immediately after handgrip exercise. Reduction in forearm blood flow by lower body negative pressure during infusion of SMTC was not significantly different from that during vehicle (−28.5 ± 4.02 vs. −34.1 ± 2.96%, respectively; P = 0.32; n = 8). However, L-NMMA augmented the reduction in forearm blood flow by lower body negative pressure (−44.2 ± 3.53 vs. −23.4 ± 5.71%; n = 8; P < 0.01). When lower body negative pressure was continued after handgrip exercise, there was no significant effect of either L-NMMA or SMTC on forearm blood flow immediately after low-intensity exercise (P = 0.91 and P = 0.44 for L-NMMA vs. saline and SMTC vs. saline, respectively; each n = 10) or high-intensity exercise (P = 0.46 and P = 0.68 for L-NMMA vs. saline and SMTC vs. saline, respectively; each n = 10). These results suggest that sympathetic activation increases NO release from eNOS, attenuating vasoconstriction. Dysfunction of eNOS could augment vasoconstrictor and blood pressure responses to sympathetic activation. However, neither eNOS nor nNOS plays an essential role in postexercise hyperaemia, even in the presence of increased sympathetic activation.

endothelial nitric oxide synthase; exercise; forearm blood flow; neuronal nitric oxide synthase; sympathetic vasoconstriction

NITRIC OXIDE (NO), SYNTHESIZED from L-arginine and molecular oxygen by NO synthases (NOS) (21–23), plays an important role in regulating arteriolar tone, peripheral resistance, and blood flow, acting as a tonic dilator opposing sympathetically mediated vasoconstriction (28). Although it has been considered that NO derived from endothelial NOS (eNOS) is the major regulator of local blood flow, it is now clear that NO synthesised from the neuronal NOS isoform (nNOS) plays an equally important role. In humans, brachial artery infusion of S-methyl-l-citrulline (SMTC), a selective inhibitor of nNOS, reduces basal forearm blood flow (FBF) but does not block the eNOS-mediated vasodilator response to ACh or increased shear stress (24, 25). This suggests that nNOS-derived NO contributes to the tonic regulation of basal arteriolar tone and hence blood flow. nNOS is expressed mainly in nonadrenergic, noncholinergic nitrergic nerves, and nNOS inhibition suppresses the vasodilatory response to mental stress (thought to be a nitrergically mediated response) (25), suggesting that nitrergic nerves might be the source of nNOS influencing basal arteriolar tone. However, other tissues expressing nNOS such as skeletal muscle (20) and vascular smooth muscle (16) could be involved.

Animal studies and indirect observations in humans suggest that sympathetic nerve activity may stimulate NO release from eNOS and/or nNOS (18, 27), and NO release by this or other mechanisms could contribute to functional sympatholysis, the local attenuation of sympathetic vasoconstriction in exercising muscle and/or to direct vasodilation after exercise. A role for NOS and particularly nNOS in functional sympatholysis is supported by some (1, 19, 26) but not all (6) animal studies. However, human studies using nonspecific inhibitors of NOS suggest the role of NOS in regulation of blood flow immediately after local exercise is small or absent (7, 8, 10, 11) but have not been performed in the presence of the increased sympathetic activation expected during systemic exercise.

We tested the hypothesis that NO release from nNOS and/or eNOS regulates local FBF during reflex sympathetic activation at rest and after exercise. We used nonselective and selective NOS inhibitors to determine the role of eNOS-derived and nNOS-derived NO in 1) opposing sympathetically mediated increases in arteriolar tone in the forearm during low body negative pressure (LBNP) applied at rest and 2) contributing to the increase in blood flow after handgrip exercise during LBNP. We also examined effects of NOS inhibition on blood flow responses after exercise without LBNP and the effects of propranolol on the blood flow response to LBNP to determine whether β-adrenergic responses are implicated in this response.

METHODS

The studies were approved by the local Research Ethics Committee (St. Thomas’ Hospital), and all participants provided written informed consent. The studies conformed to the standards set by the latest revision of the Declaration of Helsinki.

Healthy male volunteers, aged 25.9 ± 6.9 years, were recruited by local advertisement. Subjects on regular medication with hypertension, hyperlipidaemia, or any significant abnormality on hematological or biochemical screening were excluded. Participants were asked to abstain from caffeine for at least 12 h before the studies.

FBF studies were undertaken in a quiet temperature-controlled vascular laboratory (23°C to 25°C) after at least 30 min of rest. A
27-gauge needle was inserted into the brachial artery under local anesthesia by using less than 0.2 ml 1% lignocaine, and saline vehicle or drugs infused at 1 ml/min by constant rate infusion pump. FBF was measured by venous occlusion plethysmography by using electrically calibrated strain gauges (14). Drugs were infused for at least 5 min, and blood flow was measured over the final 1 min of infusion, with the mean of 5 measurements used for analysis. The protocols below were used to assess effects of the nonselective NOS inhibitor L-arginine (L-NMMA; 2 μmol/min) and the nNOS specific NOS inhibitor SMTC (0.2 μmol/min) on reflex sympathetic activation induced by LBNP and handgrip exercise (Fig. 1). These doses of L-NMMA and SMTC were chosen because they produce the same reduction in basal FBF [with effects that are near maximal within 5 min (25, 28) and that for SMTC is specific for nNOS, inhibiting nitricergic responses but being without effect on eNOS-stimulated responses]. Furthermore, these doses of L-NMMA and SMTC inhibit eNOS and nNOS and nNOS, respectively, under high-flow conditions such as during activation of eNOS by shear stress (24) or substance P (4), and nNOS by mental stress (25). SMTC at a purity >99% was obtained from Calbiochem UK and prepared to standards suitable for human use in a nationally accredited pharmaceutical manufacturing facility as previously described (25). Pharmaceutical grade L-NMMA was purchased from Bachem (Switzerland).

**Effects of SMTC and L-NMMA on FBF during sympathetic activation.** Saline vehicle was infused for 15 min to establish baseline FBF, followed by infusion of SMTC (0.2 μmol/min for 15 min; n = 8) and, in a separate group of subjects, L-NMMA (2 μmol/min for 15 min; n = 8). During the final 3 min of each infusion period, reflex sympathetic activation was induced by applying ~20 mmHg LBNP using a lower body chamber sealed above the level of the iliac crest. This level of LBNP simulates mild orthostatic stress, unloading the cardiopulmonary mechanoreceptors, leading to a reflex increase in sympathetic output and reproducible reduction in FBF (15, 31).

**Effects of β-adrenergic blockade on FBF during sympathetic activation.** After baseline FBF was established, ~20 mmHg LBNP was applied for 5 min. After return of FBF to baseline, propranolol (50 μg/min; n = 12) was infused for 5 min under basal conditions and during a further application of LBNP (~20 mmHg for 5 min).

**Effects of L-NMMA on FBF immediately after handgrip exercise.** After baseline FBF during infusion of saline was established, subjects performed handgrip exercise using a modified Grip dynamometer (US Gauge) for 3 min at 30 pulls/min at low intensity (30% maximal voluntary contraction) and for 3 min during high-intensity (80% maximal voluntary contraction) handgrip exercise. After ~25 min recovery, L-NMMA (2 μmol/min; n = 11) was infused for 7 min at rest, during further 3-min periods of low- and high-intensity handgrip exercise, and during FBF measurements immediately after exercise. These were made for 1 min immediately after the cessation of handgrip exercise with the mean of at least 5 measurements used for analysis. To control for a carry-over effect of the first period of exercise, this protocol was repeated in a separate group of subjects (n = 11) with the infusion of saline control in place of L-NMMA during the second two periods of exercise.

Fig. 1. Schematic of study protocols. Forearm blood flow (FBF) was measured by using venous occlusion plethysmography. A: effect of low body negative pressure (LBNP) on FBF was measured during infusion of NO-monomethyl-L-arginine (L-NMMA; 2 μmol/min) and vehicle, and S-methyl-L-thiocitrulline (SMTC; 0.2 μmol/min) and vehicle. B: effect of LBNP on FBF was measured during infusion of propranolol and vehicle. C: effect of low- and high-intensity hand-grip exercise on FBF was measured in the presence of L-NMMA and vehicle and also with vehicle throughout. D: effect of simultaneous LBNP with low- and high-intensity exercise on FBF was measured in the presence of L-NMMA, SMTC, and saline control in a cross-over study.
Effects of SMTC and L-NMMA on FBF immediately after exercise during LBNP. FBF remained persistently elevated after exercise despite a prolonged period of recovery. This final protocol was, therefore, performed on separate days. After baseline FBF was established, subjects had an infusion of either saline (n = 10), SMTC (0.2 μmol/min; n = 10), or L-NMMA (2 μmol/min; n = 10) for 7 min at rest, during 3-min periods of low- and high-intensity handgrip exercise, and during FBF measurements 1 min after exercise. This was done in the presence of −20 mmHg LBNP, which commenced immediately before exercise and continued after exercise during the measurement of FBF.

Statistical analysis. Data were summarized as means ± SE. Vasoconstrictor responses to SMTC, L-NMMA, and LBNP were expressed as the absolute decrease in FBF and percent reduction in FBF from baseline. Vasoconstrictor responses to exercise were expressed as the absolute FBF. This method of expressing FBF responses has previously been shown to be the most reproducible (29). Effects of the NOS inhibitors on the blood flow responses were analyzed by one-way ANOVA or by ANOVA for repeated measures as appropriate. All tests were two-tailed, and differences were considered significant when P < 0.05.

RESULTS

Propranolol does not affect the FBF response to LBNP. Propranolol was used to examine the possible contribution of β-adrenergic activation to opposing the vasoconstrictor response to sympathetic stimulation by LBNP. Propranolol had no significant effect on basal FBF (data not shown) or on the response to LBNP (−34 ± 3.5 vs. −27 ± 4.2 reduction during saline and propranolol, respectively; P = 0.21).

L-NMMA but not SMTC augments the FBF response to LBNP. L-NMMA and SMTC were used to examine the possible contributions of NOS (eNOS and nNOS) and nNOS, respectively, in opposing the vasoconstrictor response to sympathetic stimulation by LBNP. At rest and in the absence of LBNP, both L-NMMA (2 μmol/min) and SMTC (0.2 μmol/min) significantly reduced FBF by 25.8 ± 3.0% (from 2.76 ± 0.20 to 2.02 ± 0.12 ml·min⁻¹·100 ml⁻¹; P < 0.001; Fig. 2A) and 20.6 ± 3.3% (from 3.42 ± 0.31 to 2.71 ± 0.27 ml·min⁻¹·100 ml⁻¹; P < 0.01, Fig. 2B) and 20.5 ± 3.4% (from 3.42 ± 0.31 to 2.71 ± 0.27 ml·min⁻¹·100 ml⁻¹; P < 0.01, Fig. 2B) and 20.5 ± 3.4% (from 3.42 ± 0.31 to 2.71 ± 0.27 ml·min⁻¹·100 ml⁻¹; P < 0.01, Fig. 2B), respectively. LBNP (−20 mmHg) reduced FBF by 23.4 ± 5.7% (from 2.75 ± 0.20 to 2.12 ± 0.22 ml·min⁻¹·100 ml⁻¹; P < 0.01) during saline vehicle infusion. In the presence of L-NMMA, LBNP reduced flow by 44.2 ± 3.5% (from 2.02 ± 0.12 to 1.14 ± 0.11 ml·min⁻¹·100 ml⁻¹), significantly more than during saline vehicle (P < 0.01 vs. saline; Fig. 2A). By contrast, the reduction in FBF by LBNP during infusion of SMTC (28.5 ± 4.0% reduction from 2.71 ± 0.27 to 1.92 ± 0.18 ml·min⁻¹·100 ml⁻¹) did not differ significantly from that observed during saline infusion (a 34.1 ± 3.0% reduction, from 3.42 ± 0.31 to 2.24 ± 0.20 ml·min⁻¹·100 ml⁻¹; P = 0.32; Fig. 2B). Thus the relative reduction in FBF in response to LBNP was significantly greater in the presence of L-NMMA than in the presence of SMTC (44.2 ± 3.5 vs. 28.5 ± 4.0%; P < 0.01; Fig. 2C).

L-NMMA does not affect the FBF response immediately after exercise. L-NMMA was used to examine the possible contribution of NOS (both eNOS and nNOS) to the increase in FBF immediately after exercise. During saline infusion, FBF increased from a baseline of 2.70 ± 0.29 to 11.06 ± 1.16 and 17.86 ± 1.06 ml·min⁻¹·100 ml⁻¹ immediately after low- and high-intensity handgrip exercise, respectively. An increase in FBF persisted after exercise despite a 25-min period of recovery (2.78 ± 0.17 ml·min⁻¹·100 ml⁻¹ before exercise vs. 5.65 ± 0.36 ml·min⁻¹·100 ml⁻¹ after recovery). Nevertheless, the FBF elicited by the second period of low- and high-intensity handgrip in the presence of L-NMMA was not significantly different to that observed during saline infusion (Fig. 3A; P = 0.22 and P = 0.32, respectively). In the control study where saline vehicle was infused in place of L-NMMA, a similar persistent increase in FBF was seen after 25-min recovery from the first period of exercise (2.70 ± 0.29 ml·min⁻¹·100 ml⁻¹ before exercise vs. 4.97 ± 0.51 ml·min⁻¹·100 ml⁻¹ after recovery). However, the FBF elicited immediately after the first exercise periods was not significantly different to that during the second exercise periods of low- and high-intensity exercise (P = 0.73 and P = 0.63, respectively).

Neither L-NMMA nor SMTC affects the FBF response immediately after exercise during LBNP. L-NMMA and SMTC were used to examine the possible contributions of NOS (eNOS and nNOS) and nNOS, respectively, to the increase in FBF immediately after exercise in the presence of additional sympathetic stimulation with LBNP. On separate study days, L-NMMA (2 μmol/min) and SMTC (0.2 μmol/min) reduced resting blood flow to a similar degree, by 23.2 ± 2.2% (from 5.40 ± 0.72 to 4.21 ± 0.61 ml·min⁻¹·100 ml⁻¹; P < 0.001) and 19.6 ± 2.5% (from 5.46 ± 0.73 to 4.42 ± 0.60 ml·min⁻¹·100 ml⁻¹; P < 0.001), respectively (P = 0.29 for

![Fig. 2 A: FBF at rest and during LBNP during infusion of saline and L-NMMA. *P < 0.01 vs. saline with no LBNP; **P < 0.001 vs. saline with no LBNP; †P < 0.001 vs. L-NMMA with no LBNP. B: FBF at rest and during LBNP during infusion of saline and SMTC. *P < 0.001 vs. saline with no LBNP; **P < 0.01 vs. saline with no LBNP; †P < 0.01 vs. SMTC with no LBNP. C: percent change in FBF during L-NMMA and SMTC with LBNP. *P < 0.01 vs. SMTC (+, LBNP; −, no LBNP).](http://ajpheart.physiology.org/)

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ANOVA restricted to subjects that attended all study days and way ANOVA on all subjects or a repeated-measurements Results did not differ when data were analyzed using a one-

mmHg) in the presence of L-NMMA was not significantly when compared with saline, for either low-intensity exercise (P < 0.05) and high intensity, respectively), SMTC compared with saline (P > 0.05) and L-NMMA or SMTC infusion when compared with saline, for either low-intensity exercise (P = 0.91 and P = 0.44, respectively; n = 10) or high-intensity exercise (P = 0.46 and P = 0.68, respectively; n = 10).

\[ FBF = \frac{Q}{A} \]

**Fig. 3.** A: comparison of FBF after undertaking low- and high-intensity exercise, during saline or L-NMMA infusion. There was no significant difference in FBF during L-NMMA infusion when compared with saline during either low-intensity exercise (P = 0.22, n = 11) or high-intensity exercise (P = 0.32, n = 11). B: a comparison of the FBF immediately after low- and high-intensity exercise and LBNP while infusing saline, L-NMMA, or SMTC. There is no significant difference in FBF during L-NMMA or SMTC infusion when compared with saline, for either low-intensity exercise (P = 0.91 and P = 0.44, respectively; n = 10) or high-intensity exercise (P = 0.46 and P = 0.68, respectively; n = 10).

\[ \text{FBF} = \frac{Q}{A} \]

\[ \text{L-NMMA vs. SMTC} \]. The FBF in response to low- and high-intensity exercise during simultaneous LBNP (−20 mmHg) in the presence of L-NMMA was not significantly different to that during saline (P = 0.91 and P = 0.44 for low and high intensity, respectively), SMTC compared with saline (P = 0.46 and P = 0.68 for low and high intensity, respectively), and SMTC compared with L-NMMA (P = 0.31 and P = 0.76 for low and high intensity, respectively; Fig. 3B). Results did not differ when data were analyzed using a one-way ANOVA on all subjects or a repeated-measurements ANOVA restricted to subjects that attended all study days and thus had data during infusion of saline, L-NMMA, or SMTC.

**DISCUSSION**

Until recently it had been assumed that the NO that regulates local blood flow under physiological conditions in humans derives exclusively from eNOS. However, first-in-human studies with the nNOS-selective inhibitor SMTC showed that the basal regulation of vascular tone in both the forearm and coronary circulations is mediated by nNOS, whereas eNOS mediates relaxant responses to pharmacological and shear stress stimuli (24, 25). Cutaneous vasodilatation induced by heat stress is also mediated by nNOS in humans (17). The autonomic nervous system could activate local release of NO from nNOS through a neuronal link and/or eNOS through local release of neurotransmitters such as norepinephrine. To our knowledge, this is the first study to examine the role of nNOS- and eNOS-mediated vasomotor responses to physiological stimuli that modulate activity of the sympathetic and possibly other neural pathways within the autonomic nervous system. Nonisoform selective NOS inhibition with L-NMMA augmented the forearm vasoconstrictor response to LBNP, whereas selective nNOS inhibition (despite causing a similar reduction in basal FBF to that observed with L-NMMA) had no significant effect on the vasoconstrictor response to LBNP. This suggests that reflex sympathetic vasoconstriction at rest is partially opposed by eNOS- but not by nNOS-derived NO. These results are consistent with observations in the rat hind limb where nonselective NOS inhibition with \textsuperscript{N}-nitro-L-arginine methyl ester (L-NAME) increases muscle vasoconstriction in response to lumbar sympathetic nerve stimulation (27).

The simplest explanation for the augmented vasoconstriction to sympathetic stimulation after NOS inhibition is that norepinephrine released from sympathetic nerves in response to LBNP stimulates eNOS through \( \alpha \)- or \( \beta \)-adrenergic receptors, actions that have previously been described in animals and humans (18). The nonselective \( \beta \)-adrenergic receptor antagonist propranolol had no significant effect on the FBF response to LBNP, suggesting that eNOS-derived NO attenuates \( \alpha \)-adrenergic mediated vasoconstriction. It is not possible to probe this with an \( \alpha \)-antagonist since this would not distinguish between antagonism of direct \( \alpha \)-receptor mediated vasocostriction or of \( \alpha \)-receptor mediated activation of eNOS.

To our knowledge, the present results are the first in the human to demonstrate that sympathetic activation stimulates NO release from eNOS, which attenuates vasoconstriction. This finding could have important potential clinical implications with eNOS dysfunction, secondary to cardiovascular factors, leading to enhanced vasoconstriction and hence an enhanced blood pressure response to sympathetic activation.

Increases in blood flow during exercise depend upon short-term adjustments and interplay between autonomic influences and local regulatory factors. Previous human and animal studies examining the role of NO during functional hyperemia have yielded conflicting results. Many animal models do suggest a potential role for NO, and more specifically nNOS-derived NO in regulating blood flow by blunting the vasoconstrictor response to \( \alpha \)-adrenergic activation during dynamic exercise (functional sympatholysis) and/or contributing to exercise-induced vasodilation. These include a dog hind limb model of exercise (1) a rat model of exercise (5) and nNOS null mice (9, 12, 19, 26). In the \textit{mdx} mouse, a mouse model of Duchenne Muscular Dystrophy where dystrophin deficiency results in reduced nNOS expression in skeletal muscle, the normal ability of muscle contraction to attenuate \( \alpha \)-adrenergic vasoconstriction is defective (26). Kobayashi and colleagues have suggested that nNOS in skeletal muscle contributes to increased blood flow after mild exercise in mouse models (19).

In humans, a number of studies have shown a reduction in FBF during or immediately after exercise in response to nonselective NOS inhibition with L-NMMA or L-NAME (8, 10, 11, 30). However, the size of the effects has been small and, when compared with a vasoconstrictor control with similar
effects on resting blood flow, l-NMMA has been found to have no significant effect on blood flow responses during exercise (7). Chavoshan and colleagues (2) examined the effect of a reflex increase in sympathetic efferent activity (induced by LBNP) on muscle perfusion (assessed from measurement of muscle oxygenation by near infra-red spectroscopy) during exercise. Systemic NOS inhibition with l-NAME completely reversed the blunted vasoconstrictor response to LBNP in the exercising forearm. However, such results could be influenced by the reflex response to the systemic effects of l-NAME (which include a rise in mean arterial blood pressure). Dinenno and Joyner (7) examined the effects of local NOS inhibition with l-NMMA or l-NAME on FBF responses during handgrip exercise and while stimulating release of endogenous norepinephrine (by intra-arterial infusion of tyramine). Neither NOS inhibitor restored the vasoconstrictor response to local tyramine-stimulated norepinephrine release during exercise, arguing against a role for NO in functional sympatholysis (7).

Our results using l-NMMA show no significant effect of l-NMMA to blunt blood flow immediately after exercise. Similarly, even in the face of increased sympathetic stimulation with LBNP, we found no significant effect of either l-NMMA or SMTC on FBF responses immediately after exercise. These results support the findings of Dinenno and Joyner (7) by using a different sympathetic stimulus and using both nonselective and selective inhibitors of NOS. It is likely that there are multiple mechanisms involved in exercise hyperaemia and functional sympatholysis including metabolic mediators such as adenosine, ATP, potassium, hypoxia, and hydrogen ions that may link blood flow to metabolic demands (3). Nonadrenergic, noncholinergic peptides such as calcitonin gene-related protein may also be involved (13). The present results and those of other investigators showing lack of effect of inhibition of NOS on exercise-induced hyperaemia are consistent with the proposal by Clifford and Hlvesten that there is a redundancy of vasodilators contributing to exercise-induced hyperaemia where one vasoactive compound may take over when the formation of another is inhibited (3).

Limitations. It is important to note that our measurements were made immediately after exercise rather than during exercise and therefore the results do not necessarily mean that regulation of blood flow during exercise is independent of eNOS- or nNOS-derived NO. However, lack of effect of NOS inhibition after exercise is an important finding in its own right since studies in the nNOS null mouse implicate a role for nNOS in enhancing blood flow after exercise (19): our results suggest an important species difference and/or a phenotype of the nNOS null mouse that alters vascular reactivity independent of nNOS.

Our results pertain to healthy men, and cannot be extrapolated to women or subjects with cardiovascular risk factors. Due to the limited sample size we cannot exclude a small effect of inhibition of NOS on exercise-induced hyperaemia and functional sympatholysis including metabolic mechanisms in modulating microvascular and contractile function in rat skeletal muscle. Microcirculation 18: 501–511, 2011.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: H.S., M.S., S.B., N.M., B.C., A.M.S., and P.C. completed conception and design of research; H.S., M.S., S.B., and P.C. performed experiments; H.S., M.S., S.B., N.M., B.C., A.M.S., and P.C. analyzed data; H.S., M.S., S.B., N.M., B.C., A.M.S., and P.C. interpreted results of experiments; H.S. and P.C. prepared figures; H.S., A.M.S., and P.C. drafted manuscript; H.S., M.S., S.B., N.M., B.C., A.M.S., and P.C. edited and revised manuscript; H.S., M.S., S.B., N.M., B.C., A.M.S., and P.C. approved final version of manuscript.

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