Myocardial accumulation and localization of the inducible 70-kDa heat shock protein, Hsp70, following exercise

K. J. Milne, S. Wolff, and E. G. Noble. Myocardial accumulation and localization of the inducible 70-kDa heat shock protein, Hsp70, following exercise. J Appl Physiol 113: 853–860, 2012. First published July 5, 2012; doi:10.1152/japplphysiol.00131.2012.—Exercise increases the 70-kDa heat shock protein (Hsp70) in the myocardium, and this exercise-induced increase is associated with significantly improved cardiac recovery following insult. However, while heat shock has been shown to elevate Hsp70 primarily in the cardiac vasculature of the myocardium, the localization following exercise is unknown. Male Sprague-Dawley rats performed continuous treadmill running at 30 m/min for 60 min (2% incline) on either 1 or 5 consecutive days. At 30 min and 24 h following exercise, hearts were extirpated, and the left ventricle was isolated, OCT-cork mounted, and sectioned for immunofluorescence analysis. Whereas immunofluorescent analysis revealed little to no Hsp70 in control hearts and 30 min postexercise, the accumulation of Hsp70 24 h after a single exercise bout or 5 days of training was predominantly located in large blood vessels and, in particular, colocalized with a marker of smooth muscle. Furthermore, higher core temperatures attained during exercise led to more abundant accumulation in smaller vessels and the endothelium. It is concluded that the accumulation of myocardial Hsp70 following acute exercise predominantly occurs in a cell type-specific manner, such that changes in the cardiac vasculature account for much of the increase. This accumulation appears first in the smooth muscle of larger vessels and then increases in smaller vessels and the endothelium, as core temperature attained during exercise increases. This finding supports the observations after heat shock and further suggests that the vasculature is a primary target in exercise-induced cardioprotection.

Although Hsp70 has been postulated to protect a number of cells and organelles within the myocardium, previous work has suggested that the positive effects of HS may be most associated with increased Hsp70 within or localized to the endothelium and vasculature of the myocardium (2, 24). This is an important observation, given that isolated cardiomyocytes undergoing metabolic stress (9), hyperthermia (9), oxidative stress (20), and stretch (11) (all possible exercise-related stresses) readily accumulate Hsp70 mRNA or protein. Given these observations, it is a fair assumption that cardiomyocytes are a major site of Hsp70 synthesis and accumulation when total myocardial homogenates are analyzed after exercise. Although there is some evidence supporting vascular-specific Hsp70 in skeletal muscle following exercise (32, 35), the cell-specific localization of Hsp70 in the myocardium following exercise has yet to be determined. Consequently, the present study investigated the myocardial localization of Hsp70 following HS, compared with either a single bout or several days of exercise training. It was hypothesized that, in contrast to HS, the accumulation of Hsp70 after exercise would be observed in cardiomyocytes, as well as in cells specific to the cardiac vasculature.

MATERIALS AND METHODS

Animals. The present study was approved by the University of Western Ontario Council on Animal Care and was performed in accordance with the Guidelines of the Canadian Council on Animal Care. Male Sprague-Dawley rats (8 wk old) were obtained from Charles River Laboratories (St. Constant, Quebec) and housed in standard rat cages at constant temperature and humidity with a 12:12-h light-dark cycle. All rats were fed standard rat chow and water ad libitum.

Experimental protocol and groups. Rats were randomly assigned to one of three groups: control (CON, n = 8), exercised (EX, n = 24), and heat shocked (HS24h, n = 5). EX animals were killed 30 min after a single bout of exercise (EX30m, n = 8), 24 h after a single bout of exercise (EX24h, n = 8), and 24 h after the last bout of 5 days of exercise training (EX5d, n = 8). HS24h animals were killed 24 h after a single HS treatment, and CON animals were age matched at death with the treatment groups.

Exercise protocol. EX rats were familiarized to treadmill running by a light run for 10 min at 5 and 3 days before either an acute exercise bout or 5 days of exercise training on a motor-driven treadmill. The familiarization protocol does not influence myocardial Hsp70 levels. The exercise bouts consisted of continuous treadmill running at 30 m/min for 60 min (2% incline). Animals were weighed before exercise, and rectal temperature was recorded immediately before and immediately after each exercise bout. Animals were anesthetized via intraperitoneal injection of pentobarbital sodium (Somnotol; 65 mg/kg), and hearts were extirpated for analysis of Hsp70 content and localization, 30 min or 24 h after treatment, according to their respective groups. The left ventricle was isolated, and a portion to be
used for Western blotting was immediately frozen in liquid nitrogen and stored at −70°C until further analysis. The remainder of the left ventricle was mounted on cork with OCT medium, frozen in isopentane cooled in liquid nitrogen, and subsequently stored at −70°C until further analysis. CON rats were handled similarly to the EX group without being placed on the treadmill.

**HS treatment**. Rats were anesthetized with an intraperitoneal injection of pentobarbital sodium (Somnotol, 35 mg/kg) and then wrapped in a temperature-controlled heating blanket for whole body heating to 42°C. Body temperature was monitored and maintained at 42°C for 15 min with a temperature probe inserted 5 cm into the rectum. Following heating, all animals were provided water ad libitum and continuously monitored until they were completely ambulatory. Twenty-four hours following HS, animals were anesthetized via intraperitoneal injection of pentobarbital sodium (Somnotol; 65 mg/kg), and samples of the left ventricle were collected in the same manner as indicated above.

**Western blotting**. Samples (80–100 mg) of frozen left ventricles were homogenized in 10 volumes of buffer (600 mM NaCl, 15 mM Tris base, pH 7.5, 0.5% of proteinase inhibitor, 10% Nonidet P-40), centrifuged at 5,000 g for 10 min at 4°C, and the supernatant was collected as the myocardial extract. Samples were then stored at −70°C until determination of total protein concentration [Bradford protein assay (6)] and electrophoresis. Myocardial extracts (50 μg of total protein) were separated on 12% polyacrylamide gels overlaid with 4% acrylamide stacking gel (SDS-PAGE). A molecular weight standard (Bio-Rad, 161-0373) and 50 μg of myocardial extract from a HS24h animal (laboratory standard) was run concurrently on each gel for accurate determination of the proper molecular weight of Hsp70 protein and as a standard sample, respectively. After electrophoresis, proteins were transferred overnight at a constant voltage (30 V) at 4°C in transfer buffer (10% running buffer, 20% methanol in double-distilled H2O) to nitrocellulose membranes. The membranes were then incubated in 5% milk blotto powder in Tris-buffered saline and 0.5% Tween 20 (TTBS) for 1 h. Membranes were subsequently incubated in primary antibody specific to Hsp70 (anti-Hsp70 monoclonal antibody, Stressgen SPA-810) diluted 1:3,000 in 2% milk blotto powder in Tris-buffered saline for 6 h at room temperature. Membranes were washed in TTBS 3 × 5 min and then incubated in secondary antibody (goat anti-rabbit IgG-horseradish peroxidase conjugate antibody, Bio-Rad, 170-6516) and diluted 1:9,000 in TTBS with 2% milk blotto for 1 h. For the detection of Hsp70, membranes were developed with chemiluminescent detection (Amersham) and exposed to X-ray film. Densitometric analyses were performed using a computerized image analysis system (GelPro). Protein band intensities were expressed as a percentage of the band intensity in the control sample.

**RESULTS**

**Temperature and body weight measurements**. Mean temperature and body weight measurements for each group are presented in Table 1. There were no differences in body weights and resting rectal temperatures between groups. HS and exercise significantly elevated rectal temperature above resting values (P < 0.05).

**Hsp70 accumulation in cardiac tissue**. Western blotting was used to determine the relative abundance of Hsp70 in the left ventricles from all animals (Fig. 1). Minimal Hsp70 was observed in CON and EX30m ventricles. At 24 h following an acute exercise bout (EX24h), after 5 days of exercise training (EX5d), or following 15 min of HS at 42°C (HS24h), left ventricular Hsp70 levels were significantly elevated compared with CON and EX30m values (P < 0.05, Fig. 1). Moreover, Hsp70 accumulation in cardiac tissue.

[Confocal microscopy. Mounted left ventricles were cut into 15-μm-thick sections using a Leitz cryostat. Sections were adhered to glass microscope slides for 30 min at room temperature and then stored at −30°C for a maximum of 4 wk. For protein localization, sections were air dried for 30 min at 4°C, and the supernatant was collected as the myocardial extract. Samples were then stored at −70°C until determination of total protein concentration [Bradford protein assay (6)] and electrophoresis. Myocardial extracts (50 μg of total protein) were separated on 12% polyacrylamide gels overlaid with 4% acrylamide stacking gel (SDS-PAGE). A molecular weight standard (Bio-Rad, 161-0373) and 50 μg of myocardial extract from a HS24h animal (laboratory standard) was run concurrently on each gel for accurate determination of the proper molecular weight of Hsp70 protein and as a standard sample, respectively. After electrophoresis, proteins were transferred overnight at a constant voltage (30 V) at 4°C in transfer buffer (10% running buffer, 20% methanol in double-distilled H2O) to nitrocellulose membranes. The membranes were then incubated in 5% milk blotto powder in Tris-buffered saline and 0.5% Tween 20 (TTBS) for 1 h. Membranes were subsequently incubated in primary antibody specific to Hsp70 (anti-Hsp70 monoclonal antibody, Stressgen SPA-810) diluted 1:3,000 in 2% milk blotto powder in Tris-buffered saline for 6 h at room temperature. Membranes were washed in TTBS 3 × 5 min and then incubated in secondary antibody (goat anti-rabbit IgG-horseradish peroxidase conjugate antibody, Bio-Rad, 170-6516) and diluted 1:9,000 in TTBS with 2% milk blotto for 1 h. For the detection of Hsp70, membranes were developed with chemiluminescent detection (Amersham) and exposed to X-ray film. Densitometric analyses were performed using a computerized image analysis system (GelPro). Protein band intensities were expressed as a percentage of the band intensity in the control sample.

**Table 1. Body weights and exercise temperatures**

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Body Weight, g</th>
<th>Preexercise</th>
<th>Immediately Postexercise</th>
<th>Rectal Temperature, °C</th>
</tr>
</thead>
<tbody>
<tr>
<td>CON</td>
<td>8</td>
<td>338.7 ± 13.9</td>
<td>37.4 ± 0.2</td>
<td>N/A</td>
<td>38.3 ± 0.2</td>
</tr>
<tr>
<td>EX30m</td>
<td>8</td>
<td>345.5 ± 6.0</td>
<td>37.8 ± 0.2</td>
<td>40.7 ± 0.1*</td>
<td>41.2 ± 0.2*</td>
</tr>
<tr>
<td>EX24h</td>
<td>8</td>
<td>344.2 ± 4.9</td>
<td>37.4 ± 0.1</td>
<td>40.7 ± 0.1*</td>
<td>41.2 ± 0.2*</td>
</tr>
<tr>
<td>EX5d</td>
<td>8</td>
<td>342.7 ± 4.1</td>
<td>38.2 ± 0.1</td>
<td>40.3 ± 0.1*</td>
<td>40.3 ± 0.1*</td>
</tr>
</tbody>
</table>

Values are means ± SE; n, n. no. of rats. CON, control; EX30m, exercised animals killed 30 min after a single bout of exercise; EX24h, exercised animals killed 24 h after a single bout of exercise; EX5d, exercised animals killed 24 h after the last bout of 5 days of exercise training; HS24h, animals killed 24 h after a single heat shock treatment. Rectal temperature was taken before the rats were placed on the treadmill to begin running (preexercise) and then immediately after the running bout was completed (postexercise). Data represent the mean temperatures recorded before and after each acute exercise bout.

*Significantly greater than CON and preexercise/heat shock temperature, P < 0.05.
ventricular Hsp70 from HS24h animals was significantly greater than that observed in any other group ($P < 0.05$, Fig. 1).

**Immunofluorescent analysis of Hsp70 localization.** Confocal microscopy of ventricular cross sections, double labeled with antibodies against Hsp70 and vWF, an endothelial cell marker, is presented in Fig. 2. Sections incubated with secondary antibody alone are presented in Fig. 2, A–C. Immunoreactivity of vWF was evident in all experimental groups (Fig. 2, D, G, J, M, and P), indicative of the extensive vascular network in the ventricle. Although there was little or no evidence of Hsp70 immunoreactivity in CON hearts (Fig. 2, E and F), there was extensive colocalization of Hsp70 and vWF following HS in most cardiac vessels (Fig. 2, G, H, and I). Left ventricular Hsp70 immunoreactivity 30 min postexercise was negligible (Fig. 2K). In the ventricles from EX24h animals, Hsp70 immunoreactivity was partially localized to the cardiac vasculature (Fig. 2Q; see arrows). However, in most observations, immunoreactivity for Hsp70 appears to be just outside vWF immunoreactivity. Furthermore, this pattern exhibited less overlap with vWF and was less intense than that observed in HS24h animals (Fig. 2, I vs. O and R; see overlap or lack of overlap in close proximity to asterisk). Similar to EX24h, very little colocalization was observed in EX5d ventricles (Fig. 2Q).

Confocal microscopy of ventricular cross sections double-labeled with antibodies against Hsp70 and smooth muscle actin, a smooth muscle marker, is presented in Fig. 3. Sections incubated with secondary antibody alone are shown in Fig. 3, A–C. Smooth muscle immunoreactivity was clearly evident around vessels approximately greater than $20 \mu m$ in diameter that are typically representative of terminal arterioles, arterioles, and arteries (14) in all hearts (Fig. 3, D, G, J, M, and P). Little or no Hsp70 immunoreactivity was detected in CON and EX30m animals (Fig. 3, E and K). Following HS, Hsp70 immunoreactivity was detected and colocalized with smooth muscle within the myocardium (Fig. 3, H and I). Similarly, 24 h after an acute bout of exercise or exercise training (EX24h and EX5d), Hsp70 immunoreactivity was intense and colocalized with smooth muscle (Fig. 3, O and R).

During the course of these experiments, it was noted that enhanced Hsp70 immunofluorescence with exercise varied considerably between some animals. Upon further inspection, it was noted that increases in rectal temperature during the exercise session also varied, and, given recent findings (27), this might have influenced the synthesis and accumulation of Hsp70. Cross sections from animals killed 24 h after an acute exercise bout and exhibiting various postexercise temperatures (40.8, 41.2, and 41.6°C) are presented, along with images from a CON and HS24h animal in Fig. 4. Ventricular sections were double-labeled with primary antibodies to Hsp70, vWF, and smooth muscle actin. There was little or no immunoreactivity observed with Hsp70 in CON hearts (Fig. 4, A and F). Immunoreactivity of Hsp70 was evident around the vasculature 24 h following an acute exercise bout, regardless of the postexercise temperature (Fig. 4, B–D and G–I) and tended to colocalize with smooth muscle (Fig. 4, B–D). Notably, as rectal temperature achieved during exercise increased from 40.8 to 41.6°C, Hsp70 content appeared to increase and colocalize with smooth muscle in a greater proportion of vessels (Fig. 4, B–D), and immunoreactivity outside of smooth muscle was observed (see arrows, Fig. 4D). Furthermore, as the exercise temperature increased, more Hsp70 was observed to colocalize with the endothelium (Fig. 4I), but, as noted above, HS resulted in a greater accumulation of Hsp70 in endothelial cells and more extensive localization with small and larger vessels (Fig. 4J). Furthermore, it is important to note that pilot studies showed identical results of various intensities when a different antibody (polyclonal anti-Hsp70, SPA-812) and different fixative, permeabilization, and preparation procedures were used (data not shown).

**DISCUSSION**

Exercise is a powerful, biologically relevant means of protecting the myocardium against various stressors (34). One mechanism by which exercise may protect the myocardium is through the induction of HSPs, specifically Hsp70 (28), although the requirement for an increase in Hsp70 is not mandatory (12, 36). This is not surprising, given that exercise is a global stress, consisting of many secondary stressors that can induce a host of positive physiological responses and adaptations in many tissues. Moreover, exercise type, duration, and intensity will undoubtedly influence which protective mechanisms will be most expressed in various tissues. Nonetheless, HS and exercise provide myocardial protection at least partially through the induction of HSPs. Furthermore, heat-induced Hsp70 has been localized to the endothelial cells of myocardial blood vessels in animals (2, 24, 25) and in un-stressed human tissue using stringent antigen retrieval protocols (31). The present study employed fluorescent confocal microscopy to examine the localization of this cardioprotective protein after exercise. Similar to HS, it was observed that Hsp70 is primarily located in the cardiac vasculature following exercise. However, while HS results in a profound localization
Fig. 2. Confocal micrographs of ventricular sections double-labeled with primary Hsp70 antibody (green: B, E, H, K, N, Q) and primary von Willebrand factor (vWF; red: A, D, G, I, M, P) from CON (D–F), EX (J–R), and HS (G–I) animals. C, F, I, L, O, R: vWF and Hsp70. There is little to no Hsp70 immunoreactivity occurring in CON and EX30m animals (E and K). Robust Hsp70 immunoreactivity occurs 24 h following HS, with most colocalizing with smooth muscle actin (H and I). Hsp70 immunoreactivity is evident 24 h following acute EX (N) and 24 h following 5-day training (Q). Arrows help in indicating colocalization of Hsp70 and smaller blood vessels reactive for vWF. *Contrasts in reactivity overlap. Bars = 50 μm.
Fig. 3. Confocal micrographs of ventricular sections double-labeled with primary Hsp70 antibody (green; B, E, H, K, N, Q) and primary smooth muscle actin (red; A, D, G, J, M, P) from CON (D–F), EX (J–R), and HS (G–I) animals. C, F, I, L, O, R: smooth muscle and Hsp70. While CON animals demonstrate little to no Hsp70 immunoreactivity (E), there is slight evidence of Hsp70 immunoreactivity at 30 min post-EX (K). Immunoreactivity of Hsp70 is clearly evident in HS, EX24h, and EX5d animals (H, N, Q, respectively) and colocalizes with smooth muscle actin (I, O, R, respectively). Bars = 50 μm.
Fig. 4. Confocal micrographs of ventricular sections double-labeled with Hsp70 antibody and vWF or smooth muscle antibody on a CON animal (A, A', A", F, F', F") animals with various end-exercise temperatures from EX24h animals (40.8, 41.2, and 41.6°C; B-D, B'–D', B"–D"), G-I, G"–I, G"–I"), and a single HS24h animal (42°C; E, E', E", J, J', J"), Hsp70 (green; A–E) and smooth muscle (red, A–E) immunoreactivity is shown. A″–E": merged images. D, D', D": arrows indicate Hsp70 immunoreactivity occurring outside of smooth muscle in a high-temperature EX24h animal. Hsp70 (green; F–J) and vWF (red; F–J) immunoreactivity is shown, F″–J": merged images. G–I, G″–I", G″–I": arrows indicate Hsp70 immunoreactivity occurring outside of vWF in larger blood vessels. J, J', J": after HS, arrows indicate Hsp70 immunoreactivity occurring with vWF around larger blood vessels. Note that the localization of Hsp70 outside that of either vWF or smooth muscle actin is almost undetectable, but cannot be discounted in some areas (circle). Bars = 50 μm.
of Hsp70 in both the smooth muscle and endothelium, exercise appears to increase the protein primarily in the smooth muscle of larger cardiac vessels, unless higher rectal and presumably blood temperatures are reached. Similar observations were made following both a single acute bout of exercise and after 5 consecutive days of exercise training, indicating that multiple exercise bouts do not change the cellular localization of cardiac Hsp70.

HS and exercise have both been associated with improved postischemic ventricular recovery and reductions in infarct size, and, although this protection is multifaceted, it has been at least partially linked to the induction and accumulation of Hsp70 (10, 16, 30). Viable endothelium is necessary to provide adequate coronary flow to sustain normal mechanical function (3), and poor coronary flow postischemia may be a factor in the impact of this injury (7). Proper endothelial function is needed for the protective effect of heat stress on postischemic mechanical function (2). In addition, improved coronary flow during postischemic reperfusion is associated with enhanced cardiac recovery of function (1). Hence, the protection against ischemia reperfusion afforded by HS appears to occur within the vasculature of the myocardium. The accumulation of Hsp70 observed in both the endothelium and smooth muscle may protect flow through the cardiac vasculature by preventing smooth muscle cells from damage or dysfunction (18) and by reducing leukocyte-endothelial interactions (26). By protecting the integrity of the vasculature, the cardiomyocytes they feed would ultimately be spared insult. Although the present study did not examine postischemic myocardial function following exercise, the possibility that Hsp70-related cardioprotection is also partially a result of the protection of the cardiac vasculature is likely.

Interestingly, the blood vessels make up ~10% of heart volume, whereas the majority (~83%) of cells are functioning cardiomyocytes (4). Isolated and cultured cardiomyocytes have previously been shown to induce Hsp70 in response to several stimuli, resulting in subsequent cellular protection (9, 11, 17). It is important to note that, in the present study, Hsp70 was detected by Western blotting in control ventricles (Fig. 1), even though fluorescent confocal microscopy detected little to no Hsp70 immunoreactivity (Figs. 2–4). Furthermore, as noted elsewhere (8), it is difficult to rule out extravascular Hsp70 or myocyte Hsp70 after HS or exercise in some confocal images (Fig. 4). As such, it is not suggested that myocytes lack any Hsp70, or do not possess the ability to mount a stress response, but this was clearly much less than the exercise- or HS-induced vascular colocalization in every animal examined. While not supporting our hypothesis, this did support prior observations in a separate laboratory (24). This is intriguing because HS induces similar cardiovascular changes to exercise (i.e., increased heart rate, mean arterial pressure, and adrenergic drive), but the changes do not appear to be as great as observed during exercise (22, 23). Interestingly, when rectal temperature changes as a result of exercise were low, limited Hsp70 was detected by Western blot analysis (data not shown), and colocalization with any myocardial cell type was slight (Fig. 4). This indicates the importance of elevated temperature in inducing Hsp70 in the myocardium following exercise and is in agreement with the findings of others (13, 33), as well as our own (27), who have suggested that temperature may be a more important factor in stimulating the induction of Hsp70 in the heart than the work of exercise itself.

Even in those animals with the highest rectal temperatures as a result of exercise and in those after HS, Hsp70 exhibited the greatest colocalization with the cardiac vascular smooth muscle and endothelium, but was nearly undetectable in the cardiomyocytes, suggesting that cardiomyocytes and vascular-related cells exhibit different thresholds to the HS response. In fact, endothelial cells isolated from rodent hearts heat shocked to 43°C show severalfold greater accumulation of Hsp70 than isolated cardiomyocytes from the same tissue (2). While the isolated cardiomyocytes exhibited significant induction of Hsp70 in that study, albeit to a lesser extent than the endothelium (2), 43°C was a full degree higher than either the HS24h or EX animals of the present study and could explain why very little Hsp70 was observed in cardiomyocytes in the present study. Higher temperature might explain why HS (i.e., elevated core temperature to 42°C) induces widespread expression of Hsp70 in both smooth muscle and the endothelium throughout the myocardium compared with more limited immunoreactivity observed following exercise. However, during exercise, the vascular smooth muscle of the cardiac vasculature may experience greater α1-adrenergic drive (21), a known inducer of Hsp70 in smooth muscle (37), thereby contributing to the apparent lower temperature threshold of this tissue in the present study. Interestingly, this temperature threshold difference has also been observed in nerve tissue, where higher temperatures were required to induce Hsp70 accumulation in the nerve than in the surrounding myelin sheath and blood vessels (15). Early immunoreacting smooth muscle is indicative of cardiac arteries and arterioles, given the size of the blood vessels they surround (14), and would be a normal target of adrenergic stimulation.

In summary, regular exercise has been shown to reduce the risk of myocardial infarction and increase the chance of survival in humans, and it is perhaps the most widely known preventative measure for heart disease. Although there are myocardial benefits gained from exercise (5), the exact mechanism by which exercise offers its protection to the myocardium is not entirely known. Both HS and exercise provide improved postischemic recovery in rat hearts, and Hsp70 may play a role in this regard (10, 28). The present study suggests that the protection offered by Hsp70 against ischemia reperfusion injury may lie in its accumulation in the cardiac vasculature. The present study also highlights the importance of focusing on the cardiac vasculature in future studies looking at the endogenous mechanism(s) of cellular protection following exercise preconditioning of the heart.

ACKNOWLEDGMENTS
Present address of K. Milne: Faculty of Human Kinetics, University of Windsor, Windsor, ON, Canada N9B3P4.

GRANTS
Preparation of this manuscript was made possible by research grants awarded to E. G. Noble from Natural Sciences and Engineering Research Council of Canada (no. 8170-05) and the Canadian Institutes of Health Research (no. CCT-83029).

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

Author contributions: K.J.M., S.W., and E.G.N. conception and design of research; K.J.M. and S.W. performed experiments; K.J.M. and S.W. analyzed data; K.J.M., S.W., and E.G.N. interpreted results of experiments; K.J.M. and S.W. prepared figures; K.J.M. and S.W. drafted manuscript; K.J.M. and E.G.N. visited and revised manuscript; K.J.M. and E.G.N. approved final version of manuscript.

REFERENCES


