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RESEARCH ARTICLE | *Hypoxia 2017*

Acclimatization of the systemic microcirculation to alveolar hypoxia is mediated by an iNOS-dependent increase in nitric oxide availability

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Casillan AJ, Chao J, Wood JG, Gonzalez NC. Acclimatization of the systemic microcirculation to alveolar hypoxia is mediated by an iNOS-dependent increase in nitric oxide availability. *J Appl Physiol* 123: 974–982, 2017. First published March 16, 2017; doi:10.1152/japplphysiol.00322.2016.—Rats breathing 10% O_2 show a rapid and widespread systemic microvascular inflammation that results from nitric oxide (NO) depletion secondary to increased reactive O_2 species (ROS) generation. The inflammation eventually resolves, and the microcirculation becomes resistant to more severe hypoxia. These experiments were directed to determine the mechanisms underlying this microvascular acclimatization process. Intravital microscopy of the mesentery showed that after 3 wk of hypoxia (barometric pressure ~380 Torr; partial pressure of inspired O_2 ~68–70 Torr), rats showed no evidence of inflammation; however, treatment with the inducible NO synthase (iNOS) inhibitor L-N^6-(1-iminoethyl) lysine dihydrochloride led to ROS generation, leukocyte-endothelial adherence and emigration, and increased vascular permeability. Mast cells harvested from normoxic rats underwent degranulation when exposed in vitro to monocyte chemoattractant protein-1 (MCP-1), the proximate mediator of mast cell degranulation in acute hypoxia. Mast cell degranulation by MCP-1 was prevented by the NO donor spermine-NONOate. MCP-1 did not induce degranulation of mast cells harvested from 6-day hypoxic rats; however, pretreatment with either the general NOS inhibitor L-NG-monomethyl arginine citrate or the selective iNOS inhibitor N-[3-(aminomethyl) benzyl] acetamidine restored the effect of MCP-1. iNOS was demonstrated in mast cells and alveolar macrophages of acclimatized rats. Nitrate + nitrite plasma levels decreased significantly in acute hypoxia and were restored after 6 days of acclimatization. The results support the hypothesis that the microvascular acclimatization to hypoxia results from the restoration of the ROS/NO balance mediated by iNOS expression at key sites in the inflammatory cascade.

NEW & NOTEWORTHY The study shows that the systemic inflammation of acute hypoxia resolves via an inducible nitric oxide (NO) synthase-induced restoration of the reactive O_2 species/NO balance in the systemic microcirculation. It is proposed that the acute systemic inflammation may represent the first step of the microvascular acclimatization process.

alveolar hypoxia; inflammation; acclimatization; ROS/NO balance; iNOS

RATS BREATHING 10% O_2 show a rapid, systemic microvascular inflammation characterized by generation of reactive oxygen species (ROS) (40), degranulation of perivascular mast cells (36), adherence of leukocytes to the endothelium of postcapillary venules (41), and increased vascular permeability and emigration of leukocytes to the perivascular space (39). The inflammation is not initiated by the reduction of the systemic microvascular partial pressure of inspired O_2 (P_{O_2}) but rather, by the activation of alveolar macrophages by the low alveolar P_{O_2} (17). The activated macrophages release a chemokine, monocyte chemoattractant protein-1 (MCP-1; or CCl_2), into the circulation (12–14). Circulating MCP-1, in turn, triggers the degranulation of perivascular mast cells, as shown in primary mast cell cultures (13, 14) and in vivo (15); hypoxia by itself does not produce mast cell degranulation. The MCP-1-activated mast cells release renin, together with angiotensin-converting enzyme, and initiate the systemic microvascular phase of the inflammatory process (11, 18). ROS generation can be detected at three key sites of the inflammatory cascade: alveolar macrophages (14), mast cells (36), and the leukocyte-endothelial interface (40). Whereas these phenomena have been documented largely in the mesentery microcirculation, the effects of antioxidants, mast cell degranulation antagonists, and renin-angiotensin system blockers indicate that a similar sequence of events takes place in skeletal muscle (15, 17, 18).

The inflammation resolves spontaneously: rats maintained in hypoxia for 3 wk show no evidence of microvascular inflammation in the mesenteric microcirculation. In addition, further reduction of the inspired P_{O_2} does not produce an inflammatory response in these animals (41). These features indicate that a process of microvascular acclimatization has occurred during the prolonged exposure to hypoxia.

Previous data from our laboratory suggest that the generation of ROS during acute hypoxia leads to depletion of nitric oxide (NO) and an increase in the ROS/NO balance (35, 36). The present experiments were directed to test the hypothesis that the resolution of the inflammation and the resulting microvascular acclimatization are due to the restoration of this balance and that this is mediated by an increase in microvascular NO availability secondary to expression of inducible NO synthase (iNOS). This hypothesis is based on two lines of evidence: on one hand, the inflammation of acute hypoxia is blocked by administration of NO donors and of the NO precursor L-arginine (35), as well as by antioxidants, such as SOD catalase and lipoic acid (36). On the other, administration of the iNOS inhibitor, 1,4-phenylene-bis (1,2-ethanediyl) bis-isothiourea dihydrobromide, to chronically hypoxic rats induces leukocyte-endothelial adherence (LEA) in a manner similar to that seen in nonacclimatized rats exposed to acute hypoxia (41). The results of the experiments described below

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support the hypothesis that the microvascular acclimatization to hypoxia is mediated by a restoration of NO availability through expression of iNOS in alveolar macrophages and mast cells.

METHODS

The Animal Care and Use Committee of the University of Kansas Medical Center, an institution accredited by the American Association for Accreditation of Laboratory Animal Care, approved the experimental procedures described below.

The present study includes in vivo experiments in which the mesenteric microcirculation of rats was visualized using intravital microscopy and experiments carried out in suspensions of fresh peritoneal mast cells and alveolar macrophages harvested from normoxic rats and rats acclimatized to hypoxia.

Induction of prolonged hypoxia. Male Sprague-Dawley rats of 225–250 g body weight were placed in a chamber where air was circulated at a barometric pressure (PB) of 370–380 Torr, which results in an inspired P_{O_2} of ~68–70 Torr. For the studies in intact animals, the rats were maintained in the chamber for 3 wk. Later studies showed that microvascular acclimatization was essentially complete within 4–5 days, so for the studies in primary cell cultures, which were carried out later, the donor rats were maintained in the chamber for 6 days. The chamber was opened every other day to change the cages and feed and water the rats, a procedure that took ~30 min. Normoxic controls were maintained at ambient PB (730−740 Torr) in the same room.

Intravital microscopy. The procedures for intravital microscopy of the mesentery have been described in detail before (35, 36). Briefly, the rats were anesthetized with ketamine (45 mg/kg im) and atropine (0.4 mg/kg im). PE50 catheters were placed in the jugular vein and carotid artery for injection of solutions and measurement of arterial blood pressure. A tracheotomy was performed, and the animals breathed either room air or 10% O_2 through a two-way valve (2384 series; Hans Rudolph, Shawnee, KS) connected to the tracheal tube (PE240). The abdomen was opened via a midline incision, and the ileo-cecal portion of the intestine was exteriorized and mounted on a transparent plastic stage. The exposed tissue was covered with Saran Wrap. Studies in the rat cremaster muscle covered with Saran Wrap show that cremaster microvascular P_{O_2}, measured with a phosphorescence-quenching method, drops from a normoxic value of 35 ± 2 to 7 ± 2 Torr after breathing 10% O_2 (15). This suggests that the covering of the preparation with Saran Wrap prevents significant gas exchange between the tissue and the environment. Single, unbranched postcapillary venules, with a diameter of 20–40 μm, were selected for microscopic observation. Red blood cell velocity was measured with a Doppler velocimeter to calculate shear rate (41). LEA was expressed as the number of adherent leukocytes/100 μm venule length. Adherent leukocytes were defined as those remaining stationary for ≥30 s. Ruthenium red (5 mg/100 ml) was used to estimate in vivo mast cell degranulation intensity, as described before (35). At least five mast cells were analyzed in each field of observation.

The oxidant-sensitive probe dihydrorhodamine 123 (DHR) was used to estimate ROS levels in the mesentery. DHR (10 mg) was dissolved in 1 ml DMSO. This solution (60 μl) was dissolved in 1 ml saline and infused intravenously over a 2-min period. DHR fluorescence was detected using an itensified charge-coupled device camera (Hamamatsu Photonics, Shizuoka Prefecture, Japan), as described before (40). Sensitivity of the system was adjusted so that background fluorescence was reduced to a minimum, such that no difference between the microvessels and the surrounding tissues could be detected before infusing DHR. Vascular permeability was estimated from the ratio of extra-to intravascular FITC-dependent fluorescence intensity, as described before (39). FITC–albumin was injected through the jugular vein catheter, ~30 min before the initiation of the experiment.

Primary cultures of rat peritoneal mast cells. Peritoneal mast cells were harvested by lavage of the peritoneal cavity, as described before (12), in rats anesthetized with ketamine (45 mg/kg im) and atropine (0.4 mg/kg im). The peritoneal macrophages were separated from mast cells by differential centrifugation using a Percoll solution (12). Mast cells isolated by this procedure exceed 95% in purity. The separated mast cells were resuspended in 2 ml DMEM with 10% serum and plated in a T-25 sterile flask at 37°C. Degranulation of mast cells was assessed by the uptake of ruthenium red.

Primary cultures of rat alveolar macrophages. Alveolar macrophages were collected by bronchoalveolar lavage to carry out Western blots of NOS isoforms. Rats were anesthetized with 40 mg/kg ip pentobarbital sodium. Catheters were placed in the jugular vein (PE50) and trachea (PE240). After euthanasia with an overdose of pentobarbital sodium (150 mg/kg iv), the animals were exsanguinated, and bronchoalveolar lavage was performed as described previously (12). The collected fluid was centrifuged at 1,500 rpm for 10 min. The alveolar macrophages were separated by differential centrifugation using a Percoll solution (12). The purified alveolar macrophages were resuspended in 2 ml DMEM, supplemented with 10% serum, plated in a sterile flask, and placed in a 37°C incubator, equilibrated with 5% CO_2 in air for 45 min. The supernatant was discarded and replaced with serum-free DMEM.

Immunohistochemistry. Localization of iNOS protein in the mesentery of intact rats was performed using a commercial immunohistochemistry kit (Zymed Laboratories Histostain-Plus Bulk Kit; Thermo Fisher Scientific, Waltham, MA). The mesentery microcirculation of acclimatized and nonacclimatized rats was mapped using intravital microscopy so that mesenteric venules and arterioles were identified after the staining procedures were complete.

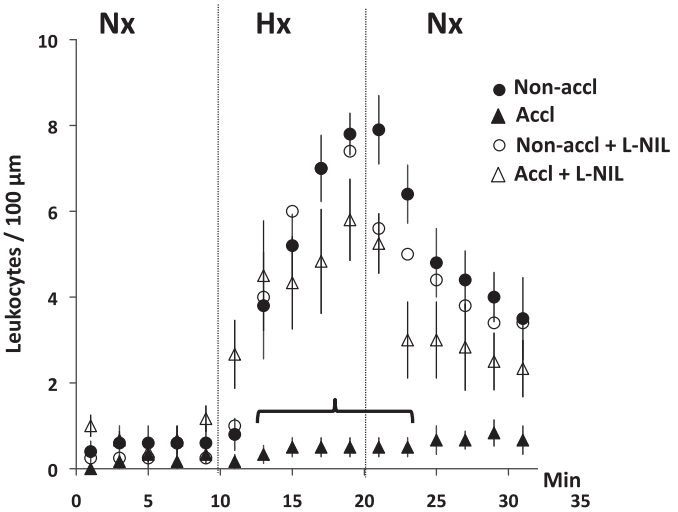

Fig. 1. Leukocyte-endothelial adherence (LEA) during room air breathing [normoxia (Nx)] or 10% O_2 breathing [hypoxia (Hx)]. During normoxia, there were no significant differences among groups. With the exception of the sample at 2 min of hypoxia, LEA was significantly lower ($P < 0.05$) during 10% O_2 breathing in the rats acclimatized for 3 wk to hypoxia (Accl) than in all of the other groups. This difference remained for the first 2 samples of the normoxic recovery period. There were no significant differences in leukocyte adherence during hypoxia or recovery among the 3 remaining groups. Values are means ± SE of 6 animals/data point. Non-accl, nonacclimatized rats; Accl, acclimatized rats; Non-accl + L-NIL, nonacclimatized rats pretreated by superfusion of the mesentery with the iNOS antagonist L-NIL (1 μM); Accl + L-NIL, acclimatized rats pretreated by superfusion of the mesentery with the iNOS antagonist L-NIL (1 μM). Superfusion was started 15 min before 0 time and maintained throughout the experiment. The acclimatized rats were exposed to room air during surgery (~30 min) and the first 10 min of the experiment.

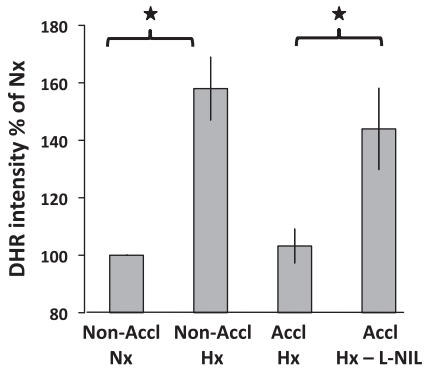

Fig. 2. Average values of DHR fluorescence intensity in nonacclimatized rats, breathing room air (Non-Accl Nx) or 10% O_2 (Non-Accl Hx). The mesentery of acclimatized rats breathing 10% O_2 was superfused with saline (Accl Hx) or with a 1-μM solution of L-NIL for 30 min (Accl Hx–L-NIL), at which time the images were obtained. Stars, significant difference ($P < 0.05$) between Nx and corresponding Hx. Values are means ± SE of 6 animals.

Mesentery tissue was then collected, and whole-mount preparations were made. iNOS was identified by the use of a primary antibody. The mesentery tissue preparations were mounted in glass coverslips and analyzed for iNOS expression by light microscopy.

Immunoblotting. Peritoneal mast cells and alveolar macrophages were collected from the culture dishes, washed with PBS, and lysed using a mammalian cell lysis kit (MCL1-1KT; Millipore Sigma, St. Louis, MO), according to the manufacturer's instructions. The Western blot membranes were probed with primary antibodies [endothelial NOS (eNOS), sc-376751; neuronal NOS (nNOS), sc-5302; iNOS, sc-8310]. Final antibody concentration was 0.1 μg/ml in all cases. Alkaline phosphatase-conjugated goat anti-mouse or anti-rabbit IgG secondary antibodies were used (1:5,000). The signals were detected using chemiluminescence (SuperSignal West Dura Chemiluminescent Substrate; Thermo Fisher Scientific). Each Western blot analysis was repeated using cells from three different donors. Densitometry was performed using ImageJ software (National Institutes of Health, Bethesda, MD).

Measurement of plasma NO metabolite levels. Plasma nitrate + nitrite concentration was measured using a colorimetric assay based on the Griess reagent (Cayman Chemicals, Ann Arbor, MI) in six animals that were placed in a chamber where 10% O_2 was circulated at ambient PB. Blood samples were obtained immediately before and at 30 and 60 min of hypoxia. The samples were obtained from carotid artery catheters placed, 48 h before the experiment, under ketamine anesthesia (45 mg/kg) and atropine (0.4 mg/kg). Blood samples were also obtained in a separate group of rats ($n = 6$) before and 6 days after being placed in the hypobaric chamber.

Statistics. Data are presented as means ± SE. Each experimental group consisted of six animals (see figure legends). Comparisons among groups were carried out with a one-way ANOVA, followed by Bonferroni tests for multiple comparisons. $P < 0.05$ was considered to indicate a significant difference.

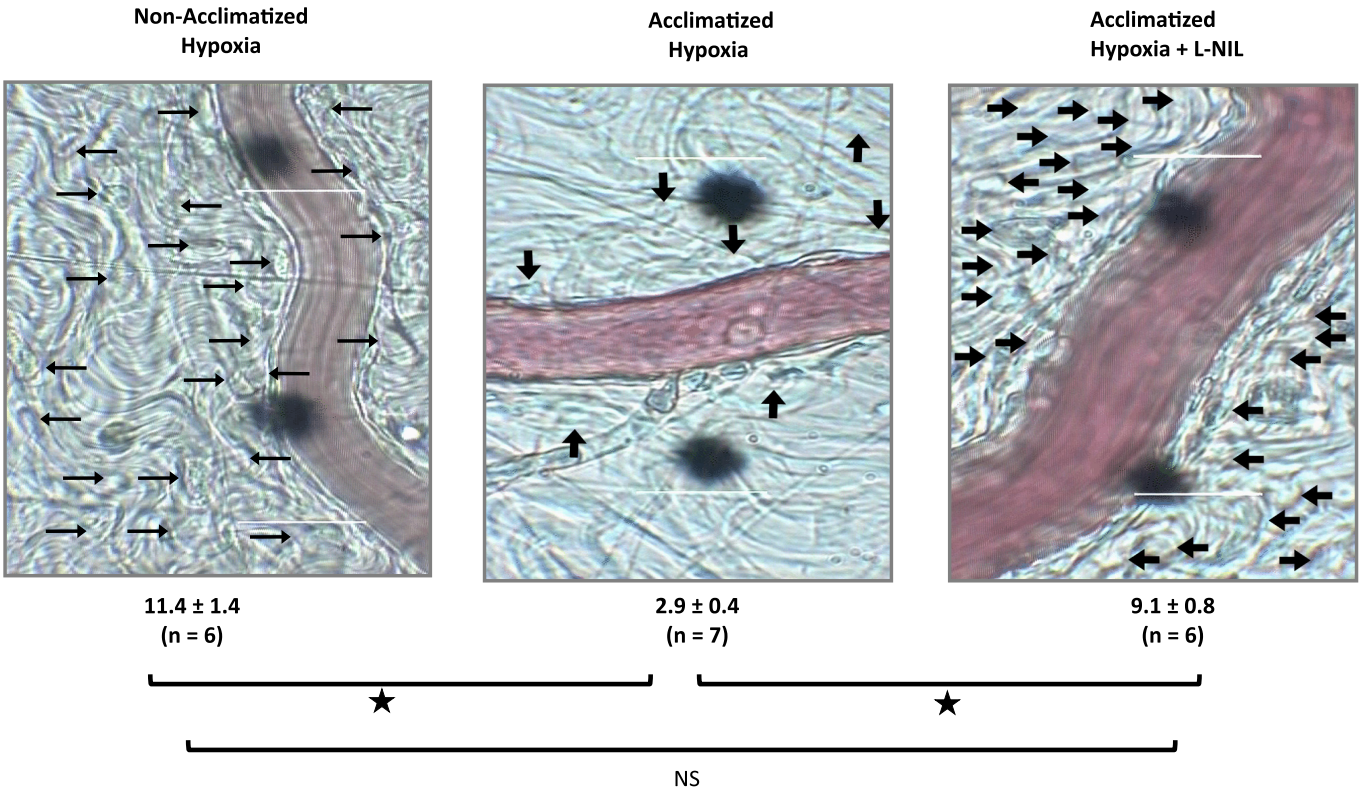

Fig. 3. Light microscopy photographs of mesenteric postcapillary venules from rats exposed to hypoxia for 4 h (nonacclimatized) or 3 wk (acclimatized), showing emigrated leukocytes identified by the arrows. All rats were maintained in a chamber, breathing 10% O_2 at PB for 4 h in a conscious state before the experiment. Breathing of 10% O_2 was continued during surgery and throughout the experiment. The animals were exposed to hypoxia for this length of time, since leukocyte emigration develops with a slower time course than leukocyte adherence to the endothelium. iNOS inhibition was achieved by intravenous infusion of L-NIL via previously implanted jugular vein catheters. A 3-mg/kg bolus of L-NIL was administered before the rats were placed in the chamber, followed by a continuous infusion (1 $mg \cdot kg^{-1} \cdot h^{-1}$) throughout the rest of the experiment. Controls received equal volumes of saline. Stars, significant difference ($P < 0.05$) between nonacclimatized hypoxia and acclimatized hypoxia and between acclimatized hypoxia and acclimatized hypoxia + L-NIL. Data below the figures are means ± SE of 6 animals.

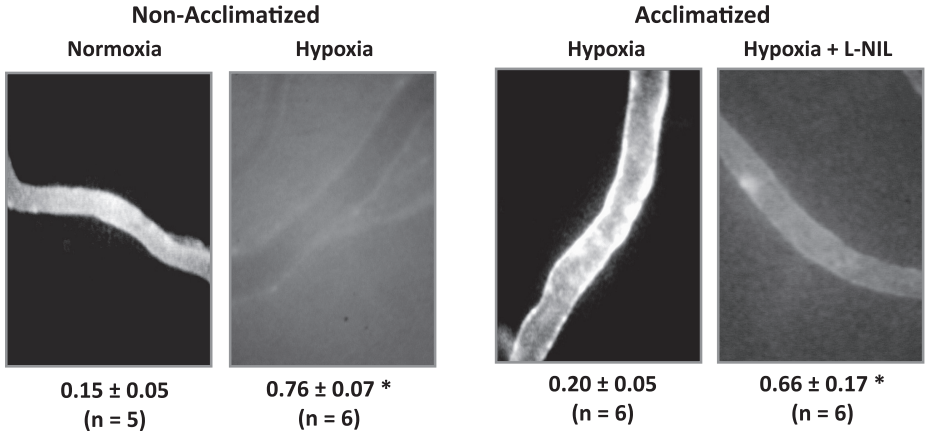

Fig. 4. Micrographs of FITC-labeled, albumin-dependent fluorescence in mesenteric venules. The nonacclimatized hypoxia rats, as well as the L-NIL-treated and untreated acclimatized rats, were maintained in a chamber, breathing 10% O_2 at PB for 4 h before the experiment. In these cases, breathing of 10% O_2 was continued during surgery and throughout the experiment. This length of exposure to hypoxia was selected, since albumin extravasation develops with a slower time course than leukocyte adherence to the endothelium. The nonacclimatized, normoxic rats were maintained in the chamber, breathing room air. The animals were exposed to hypoxia for this length of time, since increased vascular permeability develops with a slower time course than leukocyte adherence to the endothelium. A 3-mg/kg iv bolus infusion of L-NIL was administered before the rats were placed in the chamber, followed by a continuous infusion (1 $mg \cdot kg^{-1} \cdot h^{-1}$) throughout the rest of the experiment. Untreated rats received saline in the same volume. FITC–albumin was injected intravenously, 30 min before obtaining the images. *$P < 0.05$, significant difference between normoxia and nonacclimatized hypoxia and between acclimatized hypoxia and acclimatized hypoxia + L-NIL. Data below the figures (means ± SE of 6 animals) are the values of extra/intravascular FITC-dependent fluorescence intensity (36).

RESULTS

Figure 1 confirms our original observation that in contrast to nonacclimatized rats, acclimatized rats show no LEA under hypoxic conditions (41). In addition, acclimatized rats do not express other inflammatory markers, such as ROS (Fig. 2), leukocyte emigration (Fig. 3), or FITC–albumin extravasation (Fig. 4), all of which are elevated in the nonacclimatized rats breathing 10% O_2. In all cases, inhibition of iNOS using L-N^6-(1-iminoethyl) lysine dihydrochloride (L-NIL) leads to expression of all of these inflammatory markers in the acclimatized rats (Figs. 1–4). These results confirm our original observation that inhibition of iNOS using a different agent, 1,4,1,4-phenylene-bis (1,2-ethanediyl) bis-isothiourea dihydrobromide, leads to increased LEA in acclimatized rats (41) and extend these observations to the effects of iNOS inhibition on ROS generation, leukocyte emigration, and vascular permeability. Figure 5 demonstrates another aspect of microvascular acclimatization, namely, the lack of effect of H_2O_2 on LEA in the mesenteric microcirculation of acclimatized rats.

MCP-1-induced degranulation of mast cells harvested from nonacclimatized rats is blocked by the administration of an NO donor, spermine-NONOate (sNO; Fig. 6). The concentration of MCP-1 used here is the average value observed in the plasma of rats exposed to acute hypoxia (12). The same concentration of MCP-1 failed to induce degranulation of mast cells harvested from rats exposed to 6 days of hypoxia (Fig. 7); however, MCP-1 did induce degranulation when 6-day hypoxic mast cells were pretreated with either the nonspecific NOS inhibitor L-NG-monomethyl arginine citrate or with the iNOS inhibitor N-[3-(aminomethyl)benzyl]acetamidine (Fig. 7).

Figure 8 displays microphotographs of the mesentery microcirculation, demonstrating that iNOS is expressed in the peritoneal mast cells of acclimatized but not of nonacclimatized rats. This is supported by Western blot data of the three NOS isoforms in peritoneal mast cells harvested from normoxic rats and from rats exposed to hypoxia for 6 days (Fig. 9). iNOS is expressed in mast cells of rats maintained for 6 days in hypoxia but not of those harvested from normoxic rats.

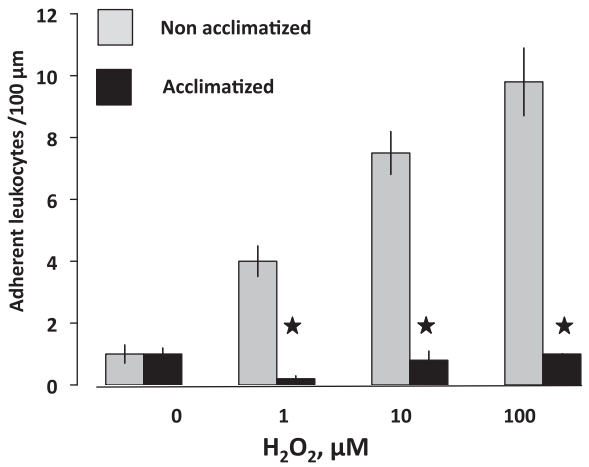

Fig. 5. Effect of superfusion of the mesentery with increasing concentrations of H_2O_2. Leukocyte-endothelial adherence was assessed 15 min after application of each H_2O_2 concentration. Stars, $P < 0.05$ between corresponding groups. Data are means ± SE of 6 animals.

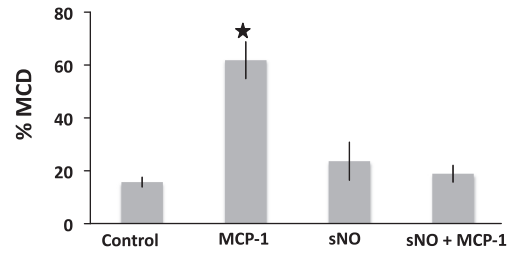

Fig. 6. Degranulation of mast cells (MCD) harvested from nonacclimatized rats in response to MCP-1 (30 ng/ml) and to the NO donor spermine-NONOate (sNO; 200 µM). MCD was assessed from the uptake of ruthenium red. Star, significant difference ($P < 0.05$) vs. control. Data are means ± SE of 5 primary cultures.

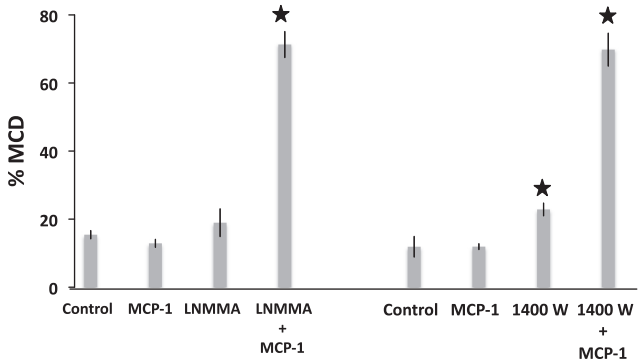

Fig. 7. Degranulation of mast cells (MCD) harvested from rats exposed to 6 days of hypoxia in response to MCP-1 (30 ng/ml). Concentration of the nonspecific NOS inhibitor L-NG-monomethyl arginine citrate (L-NMMA) was 200 μM; that of the specific iNOS inhibitor N-[3-(aminomethyl)benzyl]acetamidine (1400 W) was 200 μM. Stars, significant difference ($P < 0.05$) vs. corresponding control. Data are means ± SE of 5 primary cultures.

eNOS expression is slightly higher in peritoneal mast cells from hypoxic rats, whereas nNOS is not detectable in either group. iNOS is also expressed in the alveolar macrophages harvested from hypoxia-acclimatized rats (Fig. 10). In contrast with the effects on mast cells, acclimatization to hypoxia is accompanied by a decrease in eNOS protein expression of alveolar macrophages. nNOS is barely detectable in alveolar macrophages of either normoxic or hypoxic rats.

Plasma nitrate/nitrite concentration values (micromolars per liter) in the rats exposed to acute hypoxia were 5.95 ± 0.16 in normoxia, 4.53 ± 0.22 at 30 min of hypoxia, and 3.91 ± 0.46 at 60 min of hypoxia. The values during hypoxia were significantly lower ($P < 0.05$ and $P < 0.01$, respectively) than the normoxic value. In the rats exposed to 6 days of hypoxia plasma nitrate/nitrite, concentration was 5.09 ± 0.16 and 5.66 ± 0.26 μM/l before and at the end of hypoxic exposure, respectively (not significant).

DISCUSSION

The present results confirm our original finding that the acute inflammation resolves spontaneously during exposure to prolonged alveolar hypoxia (41) and further show that under these conditions, exogenous inflammatory agents have no effect on the microcirculation (Fig. 5). These findings are consistent with the earlier observation that the reduction of the inspired P_{O_2}, below the levels to which the rats had acclimatized, does not elicit LEA (41). The resolution of the inflammation, together with the lack of response to exogenous agents and to more severe hypoxia, can be viewed as a process of acclimatization of the systemic microcirculation to alveolar hypoxia.

Role of the ROS/NO balance in the inflammation of hypoxia and its resolution. Several lines of evidence obtained in previous studies from our laboratory indicate that the early inflammatory response is ultimately the result of an increase in the ROS/NO balance, due to depletion of microvascular NO secondary to the hypoxia-induced generation of ROS. First, microvascular DHR fluorescence intensity increases in proportion to the severity of hypoxia, as inspired O_2 is lowered from normoxia to 15, 10, and 7.5% (35). Second, there is a direct proportionality between DHR fluorescence intensity and the severity of the inflammation, as assessed by the extent of LEA (35). Finally, both DHR fluorescence and LEA are prevented by administration of antioxidants (40) or of an NO donor (35). The decrease in DHR fluorescence intensity produced by the administration of an NO donor, combined with the observation that the NO precursor L-arginine had the same effect, suggests that during hypoxia, NO was depleted and furthermore, that the depletion was the result of inactivation by ROS and not of a hypoxia-induced decrease in NO synthesis (35). The decrease in circulating NO metabolites observed in the present study is consistent with an increase in the ROS/NO balance during acute hypoxia (Fig. 11).

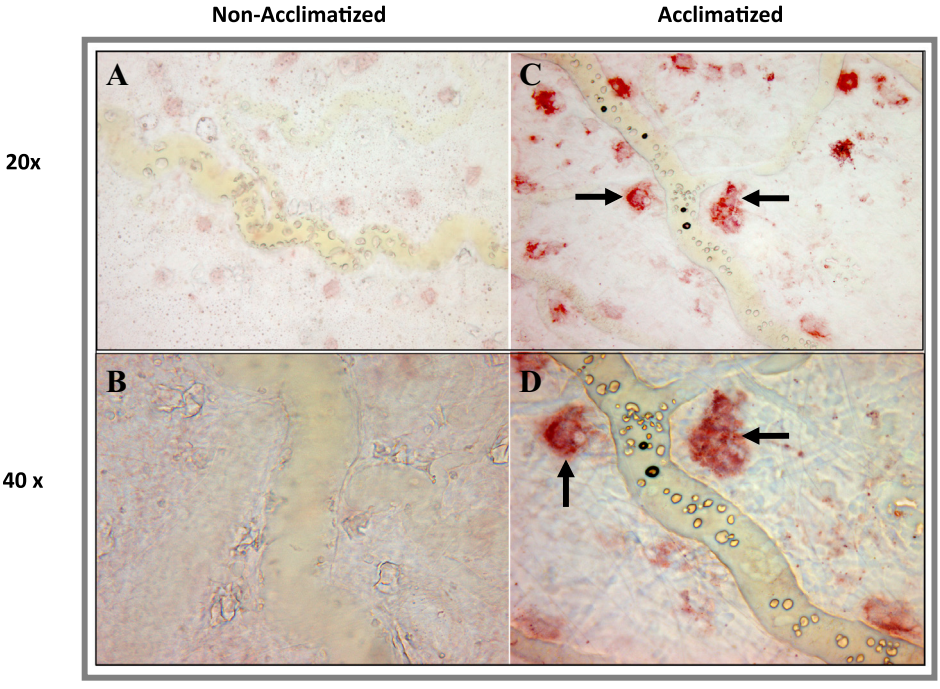

Fig. 8. Microphotographs of the mesentery microcirculation of a nonacclimatized rat (*A* and *B*) and a rat acclimatized for 3 wk to hypoxia (*C* and *D*), showing expression of iNOS using immunohistochemistry. iNOS is demonstrated in perivascular mast cells of the acclimatized rats. The arrows point to examples of peritoneal mast cells expressing iNOS. Similar results were obtained in a total of 3 acclimatized and 3 nonacclimatized rats.

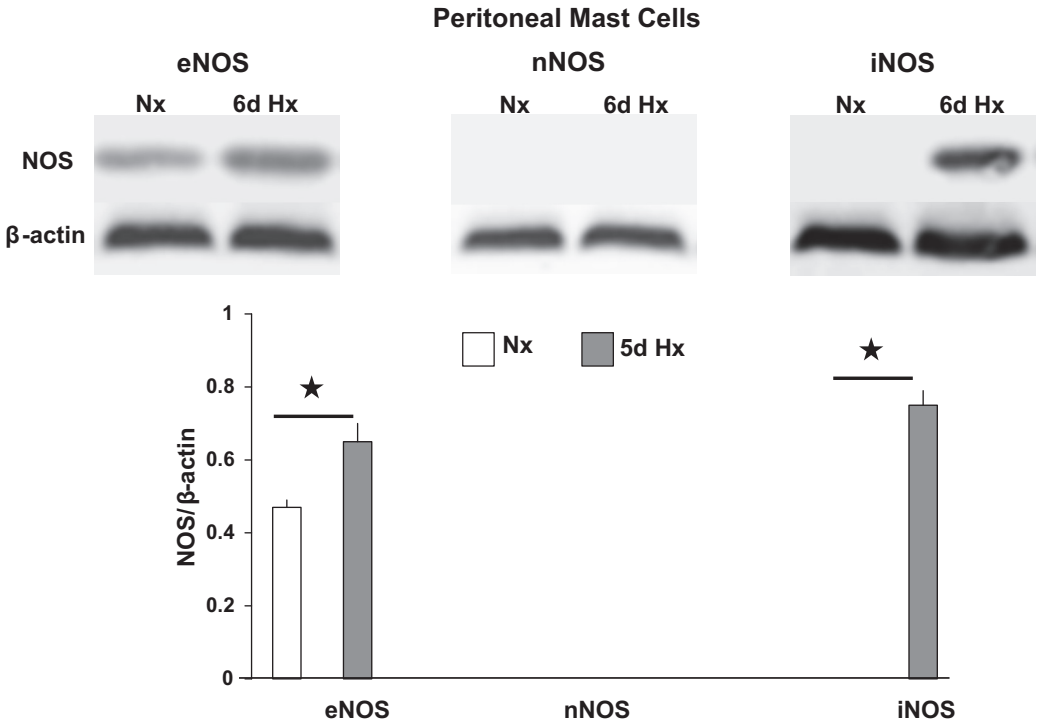

Fig. 9. Western blots of all 3 NOS in peritoneal mast cells of nonacclimatized, normoxic rats (Nx) and rats acclimatized to 6 days of hypoxia (6d Hx). Bars are densitometry averages of primary cultures of 3 rats. Stars, significant difference ($P < 0.05$) from corresponding Nx.

Against this background, the present results support the hypothesis that microvascular acclimatization involves the restoration of the ROS/NO balance and that this was achieved by a reduction of ROS and an increase in NO levels. First, the resolution of the inflammation was accompanied by a decrease in the ROS-dependent signal detected at the leukocyte-endothelial interface (Fig. 2); this coincided with a restoration of circulating levels of NO metabolites during prolonged hypoxia (Fig. 11). Whereas plasma levels of NO metabolites are determined by the global balance between NO synthesis and degradation, the pattern observed here, together with the effects of NO donors in nonacclimatized rats, is consistent with the hypothesis that the initial depletion of global NO levels during acute hypoxia was followed by an increase above preacclimatization NO levels during chronic hypoxia. For example, administration of an NO donor prevented MCP-1-induced de-

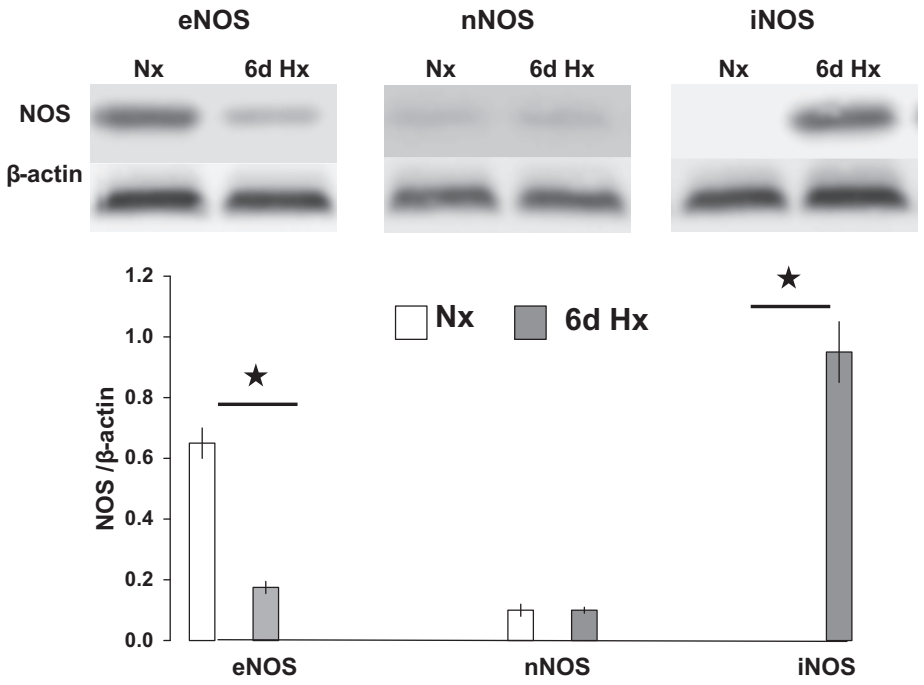

Fig. 10. Western blots of all 3 NOS in alveolar macrophages of nonacclimatized, normoxic rats (Nx) and rats acclimatized to 6 days of hypoxia (6d Hx). Bars are densitometry averages of primary cultures of 3 rats. Stars, significant difference ($P < 0.05$) from corresponding Nx.

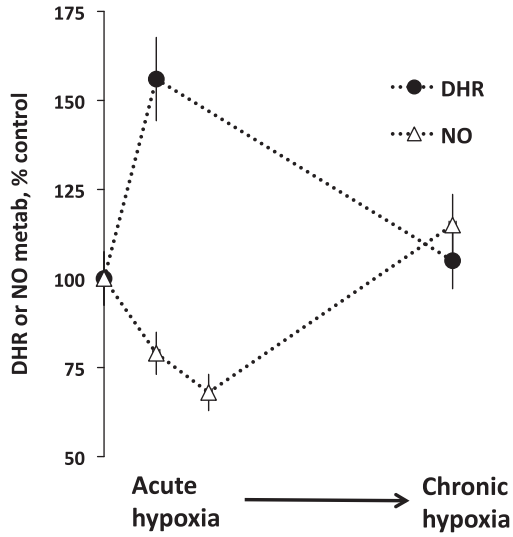

Fig. 11. Diagram of changes in DHR fluorescence intensity and plasma NO metabolite concentration in acute and chronic hypoxia. Data are expressed as percent of the normoxic value. Data for DHR fluorescence are those of Fig. 2. Plasma NO metabolite concentration values are listed in RESULTS.

granulation of mast cells from nonacclimatized rats (Fig. 6). Thus after an increase in NO concentration, mast cells of nonacclimatized rats behave as those from acclimatized rats in their response to MCP-1. This suggests that during the process of acclimatization, mast cell NO concentration increases above the preacclimatization levels. Second, several pieces of evidence suggest that iNOS played a role in the restoration of NO levels during prolonged hypoxia; iNOS expression was demonstrated in vivo in mast cells of the mesentery circulation of chronically hypoxic rats, as well as in peritoneal mast cells and alveolar macrophages harvested from rats exposed to hypoxia for 6 days. Whereas prolonged hypoxia also led to elevated eNOS expression in mast cells, it is likely that iNOS may play a greater role in increasing NO availability, given that the quantity of NO generated by iNOS is several orders of magnitude larger than that of eNOS (28). iNOS inhibition resulted in increased ROS generation and expression of inflammatory markers in the acclimatized rats (Figs. 1–4). Finally, MCP-1—the proximate in vivo mediator of mast cell degranulation during acute hypoxia—induced degranulation of mast cells of acclimatized rats in the presence of an iNOS inhibitor.

The results indicate that alteration of the ROS/NO balance of mast cells influences downstream inflammatory processes and confirm the key role of mast cells in the inflammation of hypoxia. This role is supported by the observation that activation of mast cells of normoxic rats with the secretagogue C4880 replicates the inflammation of hypoxia; conversely, the inflammation is prevented by mast cell stabilization with cromolyn (36).

The present study documents the acclimatization process in the mesentery microcirculation. The mesentery was selected for this study because this is a standard, well-characterized vascular bed, where many of the basic microvascular processes of the inflammatory response have been described. Furthermore, the mesentery is easily accessible, thus minimizing surgical trauma. We have shown that the cremaster muscle responds to acute hypoxia in a manner similar to that of the mesentery (15, 17, 18). Since the inflammation in both tissues is originated by activation of mast cells by circulating MCP-1 and since mast cells are widely distributed throughout the body, it is possible that the acute inflammation occurs in other sites besides mesentery and skeletal muscle. Whereas we have no direct evidence that other vascular beds undergo an acclimatization process similar to that seen in the mesentery, indirect evidence suggests that this may be the case. For instance, ROS generation during hypoxia can be demonstrated in alveolar macrophages (12, 13), mast cells, and at the leukocyte/endothelial interface (40), suggesting that the ROS/NO balance is also altered at these sites during acute hypoxia; the expression of iNOS in alveolar macrophages further suggests that the changes in ROS/NO balance in these cells are similar in direction as those of the mast cells. In fact, since iNOS is expressed in response to the stabilization of hypoxia-inducible factor-1α and given the widespread in vivo expression of hypoxia-inducible factor-1α during hypoxia, it is possible that the findings in mast cells are an example of a general phenomenon of the acclimatization process.

Relevance of the acute inflammation of hypoxia to altitude illnesses. The model used in these experiments approximates the conditions of high-altitude hypoxia; accordingly, it is legitimate to consider whether the inflammation contributes to the acute illnesses of altitude (6a). Of the three major high-altitude diseases—acute mountain sickness (AMS), high-altitude cerebral edema (HACE), and high-altitude pulmonary edema (HAPE)—the bulk of the evidence indicates that HAPE is secondary to excessive heterogeneous hypoxic pulmonary vasoconstriction in sensitive subjects, rather than an inflammation-induced increase in vascular permeability (29, 37).

The role of inflammation and oxidative stress in the pathogenesis of the remaining altitude illnesses—AMS and HACE—is not clear. Besides our own studies, research in animals has shown that hypoxia is accompanied by inflammation, ROS generation, and leukocyte recruitment in the brain (21, 27), mesentery, skeletal muscle (30), and isolated endothelial cells (1). Hypoxia-associated brain edema in rats was attenuated by anti-inflammatories and by interventions that increase the expression of nuclear factor (erythroid-derived 2)-like 2, a transcription factor that regulates the expression of antioxidant genes (27). In the case of humans, however, evidence for a role of inflammation and ROS generation in AMS and HACE is largely indirect and in some cases, contradictory. Gene expression of pro- and anti-inflammatory mediators correlated with the development of AMS; furthermore, acetazolamide and dexamethasone resulted in an increase in the anti-inflammatory genes in AMS-sensitive subjects (23). Similarly, the proinflammatory cytokines IL-6 and IL-8, as well as 4-hydroxynonenal, a marker of oxidative stress, are elevated in patients with AMS, whereas the anti-inflammatory cytokine IL-10 was reduced in the same subjects (34). In contrast with these results, studies using electron paramagnetic resonance imaging concluded that the mild brain volume increase associated with AMS is not the result of free radical-mediated vasogenic edema (5). Antioxidant treatment has also provided ambiguous results in the prevention or treatment of AMS (3, 6). A major difficulty in the human studies has been the uncertainty in assessing the reliability of circulating inflammatory and redox markers as indicators of processes occurring in the brain and other central organs.

An alternative role for the acute inflammation of hypoxia. The spontaneous resolution of the inflammation in virtually every case, coupled with the resistance of the microcirculation to more severe hypoxia and inflammatory agents, suggests an alternative role for the inflammation of acute hypoxia: rather than a mechanism of altitude illnesses, the inflammation may be a necessary first step in the acclimatization of the microcirculation. In this sense, the inflammation would lead to a positive outcome, with beneficial effects resulting from the increased NO availability. Higher NO levels may contribute to maintain circulatory homeostasis, prevent excessive pulmonary hypertension, and regulate vascular permeability during hypoxia. In fact, there is evidence supporting a role of increased NO availability on the effectiveness of acclimatization. Markers of NO bioavailability show a pattern similar to that seen here, with an initial decrease, followed by a secondary recovery. Plasma levels of NO metabolites decrease at 3 and 20 h of exposure to altitude (7). After acclimatization, plasma levels of NO metabolites rebound and may reach levels higher that those seen at sea level. Individuals exposed to 2 wk of altitude showed an increase in NO metabolites in plasma, saliva, and urine at *day 5* of exposure, with values declining toward control afterward (22). NO availability is greater in high-altitude dwellers than in acclimatized lowlanders. Among altitude dwellers, Tibetans, a population showing better adaptation than other altitude groups, have higher expired NO (18.6 ppb) than Bolivian Aymara highlanders (9.5 ppb) and lowlanders (7.4 ppb) (8). Conversely, some adverse effects of altitude hypoxia are accompanied by low NO levels. For example, a free radical-mediated decrease in NO levels during acute hypoxia correlates inversely with the magnitude of hypoxic pulmonary hypertension, with the greatest ROS/NO balance observed in HAPE-sensitive subjects (4). This finding is consistent with the observation that HAPE-sensitive subjects show low exhaled NO at altitude (2, 10), as well as impaired systemic endothelial function (9). Furthermore, NO inhalation improves O_2 saturation and decreases pulmonary arterial pressure more in HAPE-prone subjects than in controls exposed to the same altitude (16). In view of these results, an interesting possibility is that successful acclimatization would depend on restoration of NO availability, which is reduced by the initial inflammatory response to hypoxia.

A beneficial effect of iNOS expression. The acclimatization process described here is largely dependent on the expression of iNOS. In contrast with the beneficial effects of eNOS and nNOS on cardiovascular and nervous system function, iNOS expression has been associated with pathological effects. As pointed out before (24), this is a rather simplistic dichotomy: there are instances of positive outcomes resulting from the large amounts of NO generated by iNOS. An example is the protective effects of remote ischemic preconditioning against myocardial ischemia, which are mediated by iNOS expression (20, 33) and are absent in mice with the deleted iNOS isoform gene (19).

A beneficial effect of the systemic inflammation described here would not be a unique case of a positive outcome of an inflammatory process: in the field of hypoxia, a prominent example is ventilatory acclimatization, a phenomenon in humans and other mammals characterized by a gradual increase in ventilation over a period of days at altitude (32) and which is a key mediator of successful acclimatization (38). It has now become evident that this phenomenon is largely mediated by increased peripheral chemoreceptor sensitivity to hypoxia, which in turn, is the result of an inflammatory process characterized by macrophage invasion of the carotid body and expression of inflammatory cytokines and MCP-1 (25). Administration of ibuprofen or dexamethasone to rats exposed to prolonged hypoxia blocks the immune cell invasion, reduces the hypoxia-induced cyto- and chemokine expression in the carotid body, and prevents the development of chemoreceptor hypersensitivity during prolonged hypoxia (26, 31).

In summary, the rapid and widespread systemic inflammation of alveolar hypoxia resolves spontaneously, and the microcirculation becomes resistant to exogenous inflammatory agents and to more severe hypoxia. This phenomenon is mediated by a restoration of the ROS/NO balance via expression of iNOS in at least two key sites of the inflammatory cascade. Whereas the inflammation may contribute to the pathogenesis of some of the illnesses of altitude hypoxia, an alternative role for this process could be that of a necessary first step for the development of the acclimatization of the systemic microcirculation.

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DISCLOSURES

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

AUTHOR CONTRIBUTIONS

J.G.W. and N.C.G. conceived and designed research; A.J.C. and J.C. performed experiments; A.J.C., J.C., J.G.W., and N.C.G. analyzed data; A.J.C., J.C., J.G.W., and N.C.G. interpreted results of experiments; A.J.C., J.C., J.G.W., and N.C.G. prepared figures; N.C.G. drafted manuscript; A.J.C., J.C., J.G.W., and N.C.G. edited and revised manuscript; A.J.C., J.C., J.G.W., and N.C.G. approved final version of manuscript.

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