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Intermittent hypoxia induces NF-κB-dependent endothelial activation via adipocyte-derived mediators

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Lee MY, Wang Y, Mak JC, Ip MS. Intermittent hypoxia induces NF-κB-dependent endothelial activation via adipocyte-derived mediators. Am J Physiol Cell Physiol 310: C446–C455, 2016. First published January 6, 2016; doi:10.1152/ajpcell.00240.2015.—Aberrent release of adipocytokines from tissues dysregulates cardiometabolic functions. The present study hypothesizes that chronic intermittent hypoxia (IH) present in obstructive sleep apnea leads to adipose tissue dysfunction, which in turn contributes to vascular pathogenesis. The effect of IH was evaluated in adipose depots and aortic tissues in lean rats in vivo. Furthermore, the cellular and molecular mechanisms underlying pathophysiological interactions between adipocytes and endothelial cells were investigated in vitro. The in vivo results showed that IH induced upregulation of IL-6 and monocyte chemoattractant protein-1 (MCP-1) in IH-exposed cultured adipocytes and periaortic adipose tissues and downregulated phosphorylation of endothelial nitric oxide synthase [eNOS (ser1177)] in the aorta with activation of Erk and p38 MAPK. In support, cultured adipocytes demonstrated IH-induced elevations of NADPH oxidase 4, phosphorylation of Erk, NF-κBp65, and inducible NOS (iNOS) and increased expression of IL-6 and MCP-1. Likewise, endothelial EA.hy926 (EA) cells exposed to IH showed eNOS (ser1177) and intracellular cGMP reduction, whereas MCP-1 and iNOS expression were upregulated. Treatment of EA cells with conditioned media derived from IH-exposed cultured adipocytes caused nuclear translocation of NF-κB and augmented risk of cardiovascular morbidity and mortality (3, 18, 27). Thus, OSA represents another insult increasing the total burden of cardiovascular diseases (CVD). The endothelium plays an important role in the regulation of vascular tone and the maintenance of vascular homeostasis (27, 39). Endothelial dysfunction, characterized in vivo by a reduced flow-mediated vasodilatation attributable to decreased production of nitric oxide (NO), predicts atherosclerosis and predicts future CVD (17, 25). In patients with OSA, decreased circulating NO levels and impaired endothelium-dependent relaxations, independent of obesity, have been demonstrated (24, 27). Chronic IH during sleep may result in a number of pathological responses, such as oxidative stress, inflammation, and sympathetic activation, all of which may contribute to endothelial dysfunction (23, 24).

Adipose tissues produce an array of bioactive molecules that regulate metabolic function systemically, and, under certain pathological conditions, adipose tissues become dysfunctional and invoke downstream vascular and metabolic pathogenesis (12, 40). In animal models of diet-induced obesity, adipocytes and adherent macrophages in depot-specific adipose tissues, mainly visceral and subcutaneous fat, exhibit a state of chronic low-grade inflammation (40). Adipokines/ cytokines produced in the adipose tissues may enter into the systemic circulation to affect peripheral target organs, including the endothelium (4, 32). In particular, perivascular adipose tissue that anatomically surrounds blood vessels participates in the maintenance of vasomotor tone and the regulation of endothelial functions (5, 6). This paracrine contributory role of perivascular adipose tissue in the pathogenesis of vascular disease in humans is becoming increasingly apparent (5, 6, 8).

The present study tested the hypothesis that IH induces dysregulated release of inflammatory mediators in adipose tissues and has a pathological impact on endothelial cell activation. The release of depot-specific adipose tissue-derived proinflammatory mediators and the status of aortic activation were assessed using an in vivo lean IH-exposed rat model. The possible signaling mechanisms involved in the IH-induced activation of adipocytes and endothelial cells and their interactions were investigated using an in vitro approach.

MATERIALS AND METHODS

Animals and IH Exposure

Twelve healthy male Sprague-Dawley rats (~200 g; 6 wk old) were randomly divided into two groups and fed with standard chow diet ad libitum. One rat died during the run-in period. Rats receiving...
treatment of intermittent normoxia (IN) and IH were housed in two identical commercially designed chambers (OxyCycler model A84; Biospherix, Redfield, NY), which allowed for the application of IN or IH for 6 h daily, from 9:00 AM to 3:00 PM, for 6 wk. The oxygen (O2) concentration in the chamber was continuously measured by an O2 analyzer during the exposure. Air-exchange cycles of 2 min of 21% oxygen (room air) and 4 min of 10% oxygen were applied, resulting in alternating intervals of 90 s of 21% O2, gradually lowering to a trough of 10% O2 maintained for about 90 s toward the end of each cycle (20). This hypoxic profile was associated with arterial oxyhemoglobin saturations (SpO2), which fluctuated between a peak of 90–95%, gradually decreasing to a nadir of 60–75%, for 10 times per hour, as measured using a pulse oximeter (MouseVentG500; Kent Scientific, Torrington, CT). The achieved SpO2 pattern was similar to the reported levels in similar experimental settings (7, 13).

At the end of the 6-wk exposure, all rats were killed by an intraperitoneal overdose (100 mg/kg) of pentobarbitone. To determine whether or not chronic exposure to IH causes adipokine dysregulation in various depot-specific adipose tissues, subcutaneous, epididymal, and periaortic adipose tissues were collected and dissected in cold PBS for the determination of protein expression of proinflammatory biomarkers. Aorta with intact endothelium, which may be influenced by either circulating adipose tissue-derived factors or their perivascular adipose tissue, were snap frozen and stored at −70°C until detection of the protein expression of endothelial NO synthase [eNOS; phosphorylated (p) at ser1177], proinflammatory Erk/p38 MAPK signaling components, and inducible NOS (iNOS).

The animal care and experimental protocol for the present study were approved by the Committee on the Use of Live Animals in Teaching and Research of The University of Hong Kong (CULATR 2371-11). The research works were performed in accordance with the American Physiological Society Guiding Principles for the Care and Use of Vertebrate Animals in Research and Training.

IH Exposure and Cell Cultures

IH exposure. To evaluate the direct impact of IH on adipocytes and endothelial EA.hy926 (EA) cells, these cells were subjected separately to the exposure of IH as described (21). Briefly, cells were maintained at 37°C in 5% CO2 in a chamber (OxyCycler model A42; Biospherix) in which O2 levels were alternated between 21% for 5 min and 1% for 10 min, for a total of 64 cycles (18 h). The percentage of O2 in the cell culture medium fluctuated between 18% (normoxic phase) and a nadir of 4.5–5.0% (hypoxic phase) per cycle. Cells in the control group were maintained under normoxic condition (IN: 21% O2 and 5% CO2) throughout the experiments.

Adipogenic differentiation of preadipocytes. Primary human preadipocytes-subcutaneous (HPA-s; passages 3-5) were obtained from ScienCell Research Laboratories (cat. no. 7220; Carlsbad, CA) and cultured in Iscove’s Modified Dulbecco’s Medium (IMDM) (Sigma-Aldrich, St. Louis, MO) supplemented with 20% heat-inactivated FBS (GIBCO, Grand Island, NY). After the culture medium reached 70–80% confluence in a T25 culture flask, adipogenic differentiation was induced by replacing the culture medium with a differentiation cocktail containing 10 μg/ml insulin (GIBCO), 500 μM isobutylmethylxanthine (Sigma-Aldrich), 1 μM dexamethasone (Sigma-Aldrich), and 100 μM indomethacin (Sigma-Aldrich) in IMDM supplemented with 10% FBS for 3 days. This was followed by 2 days of maintenance in IMDM supplemented with 10% FBS and 10 μg/ml insulin. After 6 cycles of differentiation confirmed by lipid staining [Oil Red O staining (data not shown)], the cells were referred to as mature adipocytes (adipocytes). Before the experiment, adipocytes were cultured in fasting medium (3 ml IMDM containing 1% FBS and 10 μg/ml insulin) overnight before being subjected to IH. After IH exposure, cells were collected for protein extraction and evaluation of protein expressions for NADPH oxidase 4 (NOX4), Erk, p38, NF-kBp65, and iNOS. The adipocyte-derived conditioned media after IH exposure or under normoxic condition were collected, sterile filtered, and frozen at −70°C until future experiments. Levels of IL-6 and monocyte chemoattractant protein-1 (MCP-1) release in the conditioned media were measured.

Endothelial cells. Endothelial EA cells were originally obtained from American Type Culture Collection (cat. no. CRL-2922; ATCC, Manassas, VA) and were cultured in Dulbecco’s Modified Eagle Medium (DMEM GlutaMax) (GIBCO) supplemented with 10% FBS. Experiments were performed using cells at passages 6-11. Cells in six-well plates were cultured in fasting medium (1 ml DMEM containing 1% FBS) overnight before IH exposure. Treated cells were collected for protein extraction and evaluation of protein expressions for p-eNOS (ser1177), eNOS, p-Erk 1/2, Erk, p-p38, p38, and iNOS. Levels of IL-6 and MCP-1 release in the EA cell-derived conditioned media were measured.

Influence of adipocyte-derived factors on endothelial cells. To investigate the potential impact of adipocyte dysfunction on endothelial cells, conditioned media derived from cultured adipocytes under hypoxic (IH) and normoxic (IN) conditions were collected for treatment of EA cells in vitro. The conditioned media were diluted 1:1 with DMEM containing 1% FBS before treatment. Because all organs would be simultaneously vulnerable to IH exposure in vivo, all experiments were done on EA cultures under both IH or IN conditions. NF-κBp65 nuclear translocation and MCP-1 release were studied. To investigate the possible effect of IL-6 as an adipocyte-derived cytokine on endothelial cell activation, EA cells were pretreated with anti-human IL-6 antibodies (0.2 μg/ml; R&D Systems, Minneapolis, MN) or the respective control (IgG) before incubation with adipocyte-derived conditioned media. Furthermore, recombinant human IL-6 (rIL6; R&D Systems), at a concentration similar to that detected in IH-stimulated adipocyte-derived conditioned media, was added to the endothelial cells for evaluation of cGMP, MCP-1 release, and iNOS protein expression. The possible signaling mechanisms involved in endothelial cell activation by rIL6, illustrating the effect of adipocyte-derived IL-6, were determined with the use of various selective inhibitors. Inhibitors of MAPK/ERK (PD98059, 10 μM; Merck Millipore, Darmstadt, Germany), p38 MAPK (SB203580, 10 μM; Cell Signaling Technology, Danvers, MA), and NF-κBp65 kinase (SC514, 10 μM; Tocris, Avonmouth, Bristol, UK) were administered to the EA cells 45 min before IH exposure.

Intracellular cGMP Measurement

To investigate the influence of IH on NO bioavailability, intracellular cGMP ([cGMP]) levels were measured in duplicate by immunoassay (Biomedical Technologies, Stoughton, MA) in treated EA cells, according to the manufacturer’s instructions. Values were calculated as picomoles per milligram cellular protein and expressed as relative fold changes compared with the normoxic group.

Assessment of Proinflammatory Mediator Expression in Adipose Tissues or Cell Cultures

Tissue lysates from adipose tissues were prepared in ice-cold lysis buffer (Thermo Scientific, Waltham, MA) containing a cocktail of protease and phosphatase inhibitors (Thermo Scientific) and 1 mM phenylmethylsulfonyl fluoride (Thermo Scientific). The protein concentration was determined using the Bradford assay. Tissue lysates from subcutaneous, epididymal, and periaortic adipose tissues were then analyzed in duplicate for the protein expression of IL-6, MCP-1 (ELISA kits; BD Biosciences, San Jose, CA), and the content of angiotensin II (Ang II) (ELISA kit; Cayman Chemical, Ann Arbor, MI) in periaortic adipose tissues from both IN- and IH-treated groups. For in vitro experiments, the protein release of IL-6 and MCP-1 in conditioned media derived from cultured adipocytes and EA cells were analyzed in duplicate (ELISA kits; BD Biosciences) according to the manufacturer’s instructions.
Proinflammatory Mediators from Adipose Tissues in Rats

Basal and Stimulated Levels (IH Exposure) of Proinflammatory Mediators from Adipose Tissues in Rats

The basal levels of IL-6 and MCP-1 protein expression in periaortic adipose tissue were significantly higher (>10-fold) compared with that of epididymal and subcutaneous adipose samples. Exposure of the animals to IH for 6 wk significantly enhanced the protein expression of IL-6 and MCP-1 in subcutaneous and periaortic but not in epididymal adipose tissues (Fig. 1, A and B). Periaortic adipose tissue displayed a significant upregulation of Ang II and iNOS after IH (Fig. 1, C and D). However, the circulating levels of IL-6 and Ang II were below detection limits of the assays used (data not shown).

Endothelial NOS, Erk, p38, and iNOS in Rat Aorta After Exposure to IH

The rat aorta, corresponding to the site of periaortic fat depot studied, was selected for evaluation of the vasculature after IH exposure. The protein expression of one of the phosphorylation sites, ser1177, which positively regulates eNOS activity, was studied and was significantly downregulated after IH exposure (Fig. 2A). In addition, IH also induced activation of the inflammatory signaling components, p-Erk 1/2 and p-p38 (Fig. 2, B and C) in aortic tissue compared with that of IN-treated rats. The protein expression of total iNOS in aorta was not different between IN- and IH-exposed groups (Fig. 2D).

Activation of Cultured Adipocytes and Endothelial EA Cells After Exposure to IH

Exposure to IH, compared with normoxia, increased the protein expression of NOX4 in cultured adipocytes significantly (Fig. 3A). At the same time, IH also augmented the protein expressions of p-Erk 1/2, p65, and iNOS in cultured adipocytes (Fig. 3, B–D). The p38 MAPK signaling component was not changed after IH (data not shown). The levels of IL-6 and MCP-1 secreted in conditioned media from cultured adipocytes were also augmented by IH exposure (Fig. 3, E and F).

Western Blotting

Total protein lysates (30 μg) from adipose tissues, aortae, cultured adipocytes, and EA cells were separated by SDS-PAGE and transferred to nitrocellulose membranes, which were incubated overnight with the antibodies against p-eNOS (ser1177) (BD-612393), eNOS (BD-610297), iNOS (1:1,000; BD-610432; BD Biosciences), p-p38 (CS-4511), p38 (CS-9212), p-Erk1/2 (CS-4370), NF-κBp65 (CS-4764) (1:1,000; Cell Signaling), NOX4 (SC-30141; 1:500), and Erk1 (SC-94; 1:1,000; Santa Cruz Biotechnology, Dallas, TX) at 4°C. This was followed by incubation with horseradish peroxidase-labeled secondary antibodies (Novus, Littleton, CO) before image detection by enhanced chemiluminescence (Amersham Biosciences, Piscataway, NJ). A control housekeeping protein, GAPDH, was used to demonstrate the levels of sample loading.

Statistical Analysis

All data are shown as means ± SE. Statistical analysis was performed using GraphPad Prism 6.0 (GraphPad Software, San Diego, CA). Comparisons between groups were performed using independent sample t-test where appropriate. Comparisons of more than two groups were carried out with one-way ANOVA followed by Tukey’s multiple-comparisons test. P values <0.05 were considered statistically significant differences.

RESULTS

Basal and Stimulated Levels (IH Exposure) of Proinflammatory Mediators from Adipose Tissues in Rats

The basal levels of IL-6 and MCP-1 protein expression in periaortic adipose tissue were significantly higher (>10-fold) compared with that of epididymal and subcutaneous adipose samples. Exposure of the animals to IH for 6 wk significantly enhanced the protein expression of IL-6 and MCP-1 in subcutaneous and periaortic but not in epididymal adipose tissues (Fig. 1, A and B). Periaortic adipose tissue displayed a significant upregulation of Ang II and iNOS after IH (Fig. 1, C and D). However, the circulating levels of IL-6 and Ang II were below detection limits of the assays used (data not shown).
The present study demonstrates that IH exposure of lean rats induced a proinflammatory phenotype in subcutaneous and periarteric adipose tissues as well as an impaired eNOS activity with inflammatory activation in the aorta. In support of a regulatory role of adipose tissues on the endothelium, IH-exposed cultured adipocytes showed activation of NOX4 and Erk/NF-κB signaling pathways. Consequently, adipocyte-derived IL-6 was demonstrated to be a mediator in this interaction.
IH and Adipose Tissue Inflammation

The pathological impact of IH on adipose tissues has been suggested to contribute to functional alterations and upregulation of proinflammatory mediators, such as IL-6, MCP-1, TNF-α, and the oxidative product, malondialdehyde, in fat tissues of experimental animals (11, 30, 31). However, most earlier experiments focused on the evaluation of this impact on visceral fat, whereas there is limited information on other fat depots (11, 30, 31), probably because visceral fat may be more relevant for cardiometabolic diseases in humans (1, 30, 32). The present study in lean rats shows that IH induces the production of proinflammatory components (IL-6, MCP-1) in the subcutaneous fat depot. Our in vitro findings on cultured subcutaneous adipocytes also demonstrate a similar augmented proinflammatory response as evidenced by the levels of IL-6, MCP-1, and iNOS, in line with previous in vitro studies (22, 37).

NOX4, a constitutive active enzyme responsible for the generation of reactive oxygen species, is expressed in various cell types, including adipocytes (19). A significant upregulation of NOX4 protein expression was seen in cultured adipocytes after IH exposure, consistent with an increased oxidative load in IH. In line with the upregulation of NOX4 expression as the possible upstream component activating Erk/NF-κB (19, 35), the present findings demonstrate a significant increase in Erk phosphorylation and NF-κBp65 protein expressions, as well as elevation of downstream proinflammatory mediators in IH-exposed adipocytes. It is proposed that this IH-induced adipose tissue inflammation may then exert a pathological effect on the vascular endothelium.

Another notable finding relates to the differential effect of IH on various adipose tissue depots in the in vivo rat model. In the context of adipose tissue biology, the most abundant depot in human body is subcutaneous white fat, which plays an initial role in the response to external stimuli such as a positive caloric balance (1, 16). When the subcutaneous fat can no longer respond to the stimulation and becomes dysfunctional, visceral fat may be affected (1, 16). Angiopoietin-like 4, one of the key players during atherogenesis, is present in subcutaneous adipose tissue, and its level correlated with the severity of

Fig. 3. Activation of cultured adipocytes after IH. Protein expressions of NADPH oxidase 4 (NOX4) (n = 5) (A), p-Erk 1/2 (n = 5) (B), NF-κBp65 (p65; n = 4) (C), and iNOS (n = 3) (D) in cultured adipocytes exposed to IH for 64 cycles are shown. Levels of adipocyte-derived proinflammatory mediators IL-6 (n = 6) (E) and MCP-1 (n = 5) (F) in conditioned medium were measured by ELISA. Error bars are means ± SE. *P < 0.05, **P < 0.01 compared with the corresponding controls, IN. All results were normalized to the respective total protein or GAPDH and expressed as relative fold change relative to control in IN condition for Western blot experiments.
Nocturnal hypoxemia in patients with sleep apnea (9), suggesting that subcutaneous adiposity may not be entirely an innocent bystander in cardiometabolic pathogenesis in the context of IH. The absence of increase in IL-6 and MCP-1 in visceral epididymal fat in the current findings may be related to the relatively mild IH profile used. Perivascular fat tissue, with its intimate anatomic relationship with the blood vessels, is recognized as an important visceral fat tissue (38) with a distinct physiological role in the regulation of vascular function and disease (6, 8). The present data demonstrate that, compared with other adipose tissues, including visceral epididymal and subcutaneous fat, the basal and IH-induced secretion of proinflammatory mediators (IL-6 and MCP-1) is higher (by >10-fold) in the periaortic depot. These findings are in agreement with other studies under different pathological conditions, including obesity, suggesting a regulatory role of perivascular adipose tissues in vascular inflammation (6, 8). In addition, the elevated protein expression of Ang II in periaortic fat depots after IH might play a modulatory role. Besides its important role in the regulation of arterial blood pressure, Ang II induces inflammation and vascular injury (2, 28) and colocalizes with IL-6 in human coronary atherosclerotic plaques (33).

**IH and Endothelial Inflammation**

Exposure to IH has been consistently reported to result in direct activation of the endothelium and/or accelerates atherosclerosis (9, 14, 21, 23, 24, 26, 41). In the present experiments, endothelial dysfunction was observed after exposure to IH both in vivo and in vitro, as evidenced by the reduced protein expression of p-eNOS (ser1177) (aorta and cultured EA cells) and the reduced cGMP level (cultured EA cells), which is another well-established marker for NO bioavailability (10). Activation of the MAPK signaling pathway (Erk and p38) in aortic tissue with intact endothelium in vivo indicates initiation of proatherogenic responses within the vasculature (15, 26, 36). The initiation of pathological events such as upregulation of NF-κB-dependent monocyte chemotactic activity attribut-
able to reduced NO bioavailability (42) may be anticipated, given the IH-induced decrease in p-eNOS and elevation of MCP-1. Despite an increase in protein expression of iNOS in cultured endothelial cells after IH, the level of iNOS expressed in aorta was comparable between IH and control groups, in contrast to that reported in a previous animal study (14), and may be due to differences in IH duration and severity.

Adipose Tissue-Endothelium Interactions

Several studies have reported the negative impact of IH on the endothelium, as discussed (9, 14, 21, 23, 24, 26, 41). However, there are limited data regarding the regulatory impact of adipose tissues on the endothelium in the context of IH. The acceleration of atherosclerotic lesion formation in athero-
sclerotic-prone ApoE−/− mice after IH exposure can be improved after epididymal lpectomy (30). This observation suggests a possible contribution of adipose tissue dysfunction in IH-induced atherogenesis. In agreement, we found an elevation of NF-κBp65 nuclear translocation and MCP-1 release in endothelial cells after direct incubation with IH-exposed adipocyte-derived conditioned media. It has been reported previously that IL-6 participates in endothelial dysfunction and vascular pathogenesis via inflammatory activation of NF-κB signaling pathway (33, 34). The attenuation effect produced by neutralizing antibodies against IL-6 strongly supports a mediating role of IL-6 in the adipocyte-derived conditioned medium. In confirmation, the application of rIL-6 produced similar activation as IH-exposed adipocyte-derived conditioned media, which was inhibited after pretreatment with the selective inhibitors for Erk, p38 MAPK, and NF-κB, providing a mechanistic pathway for the interaction between adipocytes and endothelial cells.

Of note, the direct proinflammatory action of IH on endothelial cells was diminished in the presence of adipocyte-derived conditioned media. In IH-exposed endothelial cells, an additive increase in MCP-1 release was seen on treatment with rIL-6, but this was not observed when the cells were treated with IH-stimulated adipocyte-derived conditioned media. This suggested the possible presence of anti-inflammatory factors in adipocyte-derived conditioned media and could reflect a physiological response to mitigate the adverse impact of IH in vivo. The biological system is equipped with various homeostatic mechanisms, so it is likely that multiple mediators, pathogenetic or protective, would be at play in IH-associated atherogenesis.

Limitations and Significance

Several caveats should be noted in the interpretation of the present findings. First, using the IH model as a surrogate for OSA carries inherent limitations. The literature on IH models...
comprises diverse IH profiles, and this could result in different metabolic responses, making comparisons of data between studies difficult. However, this may be analogous to the possible human scenario of OSA, which encompasses a wide range of IH profiles, which may even vary from night to night within the same individual. There is further limitation in replicating the IH profile of the in vivo model for in vitro experiments. Nevertheless, our in vitro model can achieve swings in partial oxygen pressure in the medium for each IH cycle. Furthermore, IH is only one factor, albeit a prominent one, in OSA, which carries multiple pathophysiological factors, including hypoxia, oxygen swings, sympathetic activation, cerebral arousals, and sleep fragmentation. Second, in the present study, we have only used cultured subcutaneous adipocytes in vitro for further interrogation of adipose tissue-endothelium interactions. We have also assumed that adipocytes form the predominant cell type in adipose tissue in the lean rat and account for the major effects of such tissues. We did not exclude the contribution of activated macrophages (especially in the context of obesity) in adipose tissue inflammation. Finally, the present study selected IL-6 and MCP-1 as the potential adipocyte-derived candidates for initiation of endothelial dysfunction in the context of IH. Nevertheless, it is plausible that other adipose tissue-derived mediators may also be involved.

In summary, the present study demonstrates a pathological impact of IH on adipose tissue biology and the possible cross talk between adipocytes and endothelial cells (Fig. 8). Adipose tissue may exert its effect on the endothelium either from a distance through the blood circulation or from a perivascular depot through paracrine effects. It is conceivable that IH-induced adipose cellular dysfunction may contribute to a proatherogenic milieu and further promote inflammatory endothelial activation in the context of clinical conditions with intermittent hypoxia, such as sleep apnea.

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DISCLOSURES

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

AUTHOR CONTRIBUTIONS

Author contributions: M.Y.L., J.C.M., and M.S.I. conception and design of research; M.Y.L. and Y.W. prepared figures; M.Y.L. and J.C.M. drafted manuscript; M.Y.L., Y.W., J.C.M., and M.S.I. interpreted results of experiments. M.Y.L. and J.C.M. performed experiments; M.Y.L., Y.W., J.C.M., and M.S.I. analyzed data; M.Y.L., Y.W., J.C.M., and M.S.I. interpreted results of experiments; M.Y.L. and J.C.M. drafted manuscript; M.Y.L., J.C.M., and M.S.I. approved final version of manuscript.

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