Early effects of high-fat diet on neurovascular function and focal ischemic brain injury

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Submitted 14 November 2012; accepted in final form 22 March 2013

Obesity is an independent risk factor for acute ischemic stroke (AIS) (19, 36). An alarming recent report showed that the prevalence of AIS dramatically increased in children and young adults, which positively correlated with increases in risk factors including obesity, lipid disorders, and diabetes (13). Clinical studies also suggest that obesity is an independent predictor of unfavorable functional outcome and mortality in AIS patients treated with tissue plasminogen activator (tPA), the only therapeutic option these patients have (39, 40). Given that stroke is the leading cause of disability and that the obesity epidemic is on the rise these clinical and social problems are expected to get worse, and therefore early interventions are necessary. While experimental studies in genetic or diet-induced obesity models have shown increased cerebral infarct size and poor outcomes of stroke (7, 25, 32, 33, 41), the early impact of a high-fat diet (HFD) before the development of obesity on AIS injury and functional outcomes is not known.

It is known that the brain relies heavily on constant blood flow for proper function. Two important mechanisms that contribute to the regulation of cerebral blood perfusion are autoregulatory behavior of cerebral vessels and functional hyperemia upon increased neuronal activity (11, 16, 20). HFD can negatively affect vascular function, as demonstrated by increased myogenic tone and endothelial dysfunction in diet-induced as well as genetic models of obesity (7, 8, 24, 33). The effect of a HFD on neurovascular coupling and cerebrovascular reactivity after an ischemic insult especially in the absence of metabolic abnormalities is unknown. To address this key deficit in our knowledge, the present study tested the hypotheses that HFD 1) impairs neurovascular coupling, 2) causes cerebrovascular dysfunction, and 3) worsens short-term outcomes after cerebral ischemia.

METHODS

Animals. This study was conducted in accordance with the National Institutes of Health guidelines for the care and use of animals in research and was approved by the Division of Laboratory Animal Services at the Georgia Health Sciences University. Male Wistar rats (Harlan Laboratories, Indianapolis, IN; 4–5 wk old, n = 64) were fed either an isocaloric control diet (CD, 10% fat) or a HFD (45% fat; Research Diets, New Brunswick, NJ) for 8 wk ad libitum. Blood pressure was measured by tail cuff (Kent Scientific, Torrington, CT), and blood glucose levels were measured with a glucometer (FreeStyle, Abbott Diabetes Care, Alameda, CA).

Metabolic parameters. At death, blood was collected and processed for plasma analyses. Adipose tissue from the subcutaneous, peritoneal, and epididymal depots was collected and weighed separately. Total adiposity (all depots combined) was normalized to body weight and expressed as percent body weight. Plasma insulin (ALPCO Diagnostics, Salem, NH), triglycerides, and cholesterol (Wako USA, Richmond, VA) were measured.

Measurement of functional hyperemia. Functional hyperemia was assessed 2 days prior to ischemia injury by measuring the cerebral blood flow (CBF) change in the somatosensory cortex upon whisker stimulation (21, 22). Animals were anesthetized with ketamine-xylazine (100 and 10 mg/kg) injection, and trimmed contralateral whiskers were gently stroked at a frequency of 2.5 Hz with a cotton tip attached to a vortex. The PIM3 laser Doppler scanning system (LDS, Perimed,
Ardmore, PA) was programmed to scan an area covering somatosensory cortex, which is supplied by the middle cerebral artery (MCA), without tissue contact. CBF changes were expressed as percent increase relative to resting levels.

**Brain slice preparation.** Parenchymal arteriole (PA) function was assessed with a well-established brain slice preparation (4, 5, 15). After death, the brain was removed and 300-μm-thick coronal slices were cut in ice-cold artificial cerebrospinal fluid (aCSF) containing (mM) 3 KCl, 120 NaCl, 1 MgCl\(_2\), 26 NaHCO\(_3\), 1.25 NaH\(_2\)PO\(_4\), 2 CaCl\(_2\), 10 glucose, and 0.4 \(L\)-ascorbic acid, equilibrated with 95% \(O_2\)-5% CO\(_2\) (3). Ascorbic acid was added to reduce cell swelling (mM) 3 KCl, 120 NaCl, 1 MgCl\(_2\), 26 NaHCO\(_3\), 1.25 NaH\(_2\)PO\(_4\), 2 CaCl\(_2\), 10 glucose, and 0.4 \(L\)-ascorbic acid, equilibrated with 95% \(O_2\)-5% CO\(_2\) (3). Ascorbic acid was added to reduce cell swelling

**Video microscopy.** Diameter changes in cortical arterioles (<30-μm internal diameter) were recorded with an upright Zeiss Axioscope 2FS microscope (Carl Zeiss USA, Thornwood, NY) equipped with infrared-differential interference contrast (IR-DIC) optics, a water-immersion objective, and an EMCCD camera (iXon+885, Andor Tech, South Windsor, CT). Images were acquired at 1 frame/s and visualized and stored with IQ software (Andor Tech). The slices were perfused with aCSF (35 ± 2°C) gassed with 95% \(O_2\)-5% CO\(_2\) and were allowed to equilibrate for 10 min prior to the beginning of recording. Only one arteriole per slice was recorded. Slices were perfused with the thromboxane \(A_2\) receptor agonist U-46619 to induce vasoconstriction, and test solutions were applied in the constant presence of U-46619 after a stable preconstriction was attained. Vessels that did not respond to U-46619 were not included in the analysis. Data from arteriolar diameter (IR-DIC) experiments were analyzed with custom software created by Dr. Adrian D. Bonev (Univ. of Vermont). Changes in the internal (luminal) diameter of arterioles were determined from averaged measurements taken from multiple points across the arteriolar lumen. Baseline diameter (represented as 100%) was determined during the first ~10 min of sampling, before any experimental stimulation. All arteriolar diameter values are expressed as percentage relative to baseline. Vascular tone is expressed as “degree of constriction” relative to baseline.

**Models of ischemia.** Focal cerebral ischemia (FCI) was induced by transient MCA occlusion (MCAO) as previously described (10). Briefly, all animals were anesthetized with 2% isoflurane via inhalation. The right MCA was occluded for 3 h with a 19- to 21-mm 3-0 surgical nylon filament, which was introduced from the external carotid artery lumen into the internal carotid artery to block the origin of the MCA. The rectal temperature was maintained at 37°C with a heating pad (Fine Science Tools, Foster City, CA). The cerebral perfusion was monitored with LDS to confirm successful occlusion or reperfusion. In a subset of animals, global cerebral ischemia (GCI) was induced (10-min occlusion, 7-day reperfusion) as an alternative method of ischemia. For GCI, all animals (except sham control animals) underwent four-vessel occlusion performed as described previously (43). Briefly, 24 h after electrocautery of the vertebral arteries, the common carotid arteries (CCAs) were occluded with aneurysm clips to induce 10-min forebrain ischemia. Animals that lost their righting reflex within 30 s and whose pupils were dilated and unresponsive to light during occlusion were selected for the experiments. The clips were then removed, and the blood flow through the CCAs was confirmed before the wound was sutured. The animals of the sham group underwent identical procedures except that the CCAs were not occluded. Rectal temperature was maintained at 36.5–37.5°C throughout the experiment with a thermal blanket.

**Isolated vessel studies.** At 24 h after FCI, 2-mm basilar artery segments were isolated and mounted for myograph for isometric tension recordings as described previously (27). Concentration-response curves to serotonin [5-hydroxytryptamine (5-HT), 1 nM to 100 \(\mu\)M], endothelin-1 (ET-1, 0.01 nM to 0.1 \(\mu\)M), and the stable analog of the endoperoxide prostaglandin \(H_2\) (U-46619, 0.1 nM to 10 \(\mu\)M) were performed to evaluate vascular contractility. Endothelium-dependent relaxation to acetylcholine (ACH, 1 nM to 1 \(\mu\)M) was assessed after vessels were constricted to 60% of the baseline tension with phenylephrine (PE). Sensitivity (EC\(_{50}\)) and maximum response (R\(_{max}\)) values were calculated from the respective concentration–response equations (27).

**Evaluation of O-GlcNAcylation.** O-GlcNAcylation-modified protein levels in the basilar arteries were determined by immunoblotting as previously described (28) with anti-O-GlcNAc antibody CTD 110.6 (1:2,000; Pierce Biotechnology, Rockford, IL). All membranes were stripped and rebotted with anti-actin antibody to ensure equal protein loading.

**Infarct, edema, and hemorrhagic transformation analysis.** Brains from animals that died overnight after MCAO before euthanasia at 24 h were not processed for evaluation of ischemic injury but were included in the mortality data. The infarct volume was measured after 2,3,5-triphenyltetrazolium chloride (TTC) staining as described previously (10). Edema is reported as percent increase in ischemic hemisphere size versus the contralesional hemisphere. After staining, the hemispheres were separated and deep frozen for tissue hemoglobin (Hb) quantification with a QuantiChrom kit (BioAssay Systems, Hayward, CA) (35). Hemorrhagic transformation (HT) occurrence rate (presence of macroscopic bleeding) and severity (excess Hb, \(\mu\)g/mg protein in the ischemic hemisphere) are reported.

**Terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick end staining.** Terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick end (TUNEL) staining was performed on the free-floating coronal sections of GCI groups at 7 days after reperfusion with the In Situ Cell Death Detection Kit (Roche Diagnostics, Indianapolis, IN) as described previously (43). Samples were analyzed with a LSM510 Meta confocal microscope. For quantitative analyses, the number of TUNEL-positive cells per 250-μm length of medial CA1 pyramidal cell layer was counted bilaterally in four or five sections per animal to provide a single value for each animal. A mean ± SE was calculated from the data.

**Behavioral measurements.** Neurobehavioral tests were assessed and scored in a blinded fashion by video recording before MCAO surgery and before death in each animal. The items tested in Bederson’s score included 1 spontaneous ipsilateral circling, graded from 2 to 0; 2 hindlimb retraction and 3 forelimb flexion, graded from 1 to 0, respectively; and 4 resistance to push, graded from 1 to 0. Beam walking ability tested the stability of the animal traversing a 2.4-cm wide, 80-cm-long beam and was graded on a 7-point scale. A composite score was given by combining all the above tests, with a greater score representing better neurological outcome. Grip strength was measured with a standard grip strength meter (Columbus Instrument, Columbus, OH). The rat was gently held with its forepaws grasping the pull-bar and then pulled back consistently. The digital recording obtained from three trials was averaged.

**Statistics.** Data are expressed as means ± SE or as scatterplots with median where appropriate. Contractile responses were calculated as a percentage of KCl (120 mM)-induced contraction. Concentration-response curves were fitted with a nonlinear interactive fitting program (GraphPad Software, La Jolla, CA), and two pharmacological parameters were obtained: the maximal effect generated by the agonist (or E\(_{max}\)) and −log \(EC_{50}\) (or \(pD_2\)). Statistical analyses of E\(_{max}\) and \(pD_2\) values as well as infarct size, Hb, grip strength, and percent change in CBF were performed with Student’s t-test. HT occurrence was compared by \(x^2\)-test, and neuronal deficit by composite score was analyzed with the Mann-Whitney test. For analysis of mortality, Fisher’s exact test was used. Values of \(P < 0.05\) were considered statistically significant.
In agreement with our previous observations, reduced arterial tone such as that observed in the HFD group resulted in a reduced vasodilatory response to 10 mM K⁺ (Fig. 1C).

Third, the contractile and dilatory responses of basilar arteries before and after focal ischemic injury were determined. There was no effect of HFD on these functions if the animals were not subjected to stroke (data not shown). However, when basilar arteries were tested at 24 h after MCAO, the concentration-response curves to several vasoconstrictors including 5-HT, ET-1, and U-46619 were left-shifted, indicating enhanced sensitivity, as well as greater maximum responses (Fig. 2, A–C). Endothelium-dependent relaxation was also significantly impaired in the HFD group (Fig. 2D). O-GlcNAc levels in the basilar arteries of HFD-fed animals were significantly greater, suggesting that this posttranslational modification can be the underlying mechanism of increased contractility in basilar arteries (Fig. 3).

**Effect of HFD on cerebrovascular function.** The effect of HFD on cerebrovascular function was assessed by several methods looking at vessels of different caliber. First, functional hyperemia was measured to evaluate the response of smaller arterioles by using the relative change in CBF upon neuronal stimulation. As shown in Fig. 1, A and B, HFD animals displayed blunted change in CBF, indicating impaired neurovascular coupling.

Next, the tone and relaxation properties of PAs were measured in slice preparations. We previously showed that the degree of tone in PAs dictates the polarity of the vascular response to vasoactive signals released by activated astrocytes, with decreased tone favoring constrictions and increased tone favoring dilations (3). To determine whether the HFD induced any change in vascular tone, cortical PAs were exposed to 150 nM U-46619 to induce arteriolar constriction. While no statistically significant differences were achieved, arterioles from the HFD group showed lower baseline tone (23.9 ± 4.8%, n = 11) compared with the control group (35.7 ± 9.9%, n = 6). The values of the control group were comparable to those previously reported by us in Sprague-Dawley rats fed chow diet (3). In agreement with our previous observations, reduced tone such as that observed in the HFD group resulted in a reduced vasodilatory response to 10 mM K⁺ (Fig. 1C).

**RESULTS**

**Metabolic parameters.** Eight-week HFD significantly increased body weight and adiposity without affecting plasma lipids (Table 1). Adipose tissue in all depots (subcutaneous, peritoneal, and epididymal) was increased. There were no differences in blood glucose, blood pressure, or plasma insulin levels.

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HFD on functional outcome after ischemia-reperfusion. A composite score derived from Bederson’s score and behavioral tests compared with the CD group (Fig. 6A; 7.8 ± 1.3 in CD vs. 4.0 ± 0.8 in HFD, P < 0.05). However, there was no difference on grip strength (Fig. 6B; 1.11 ± 0.07 kgF in CD vs. 0.90 ± 0.08 kgF in HFD, P = 0.07).
This study provides novel information about the early impact of HFD on cerebrovascular function and stroke outcomes in the absence of overt metabolic changes. First, HFD impairs communication between neurons and penetrating arterioles even in the absence of an ischemic insult. Second, ischemic injury serves as a second hit and causes large-artery dysfunction in stroked HFD rats that is not otherwise detectable in HFD-alone animals. Third, stroke in HFD-fed animals that do not have obesity or metabolic derangement worsens neurovas-

![Graph A](image1)

**Fig. 2.** Effect of HFD on basilar artery function after focal cerebral ischemia [middle cerebral artery occlusion (MCAO)]-reperfusion. HFD increased the contractile response to multiple agonists (A–C) and also reduced endothelium-dependent relaxation (D). Experimental values of contraction were calculated relative to the contractile response produced by 120 mM KCl, which was taken as 100% \( n = 8 \) group. Values are means ± SE. \( * P < 0.05 \).

**DISCUSSION**

This study provides novel information about the early impact of HFD on cerebrovascular function and stroke outcomes in the absence of overt metabolic changes. First, HFD impairs communication between neurons and penetrating arterioles even in the absence of an ischemic insult. Second, ischemic injury serves as a second hit and causes large-artery dysfunction in stroked HFD rats that is not otherwise detectable in HFD-alone animals. Third, stroke in HFD-fed animals that do not have obesity or metabolic derangement worsens neurovas-

![Graph B](image2)

**Fig. 3.** O-GlcNAcylation in basilar artery after focal cerebral ischemia-reperfusion. An increase in total O-GlcNAc-protein content was seen in the HFD-fed group after MCAO \( n = 6 \) group. A representative Western blot image of O-GlcNAc-modified proteins and actin control is given in A, and cumulative data are summarized in B. Representative images were selected from the same membrane, and splices are indicated by dashed lines. Values are means ± SE. \( * P < 0.05, n = 4 \).
cular injury and functional outcomes. Collectively, these data suggest that detrimental effects of HFD start early in the disease process and preventive measures should be implemented as early as possible.

HFD or obesity is a major risk factor for vascular dysfunction. It was realized several decades ago that high intake of saturated fat in the diet significantly enhanced the development of the atherosclerotic and autoimmune lesions in aorta of the autoimmune-prone B/W mice, which were known to develop severe glomerulonephritis and vasculitis (12). Numerous studies thereafter demonstrated that HFD impaired the structure and function and increased the lesion in different vascular beds (18, 31, 37, 42). Recent studies that focused on the cerebral vasculature have found that the dilator response to ACh was impaired in cerebral arterioles of HFD-fed apoE−/− mice (24) or in basilar artery of HFD-fed peroxisome proliferator-activated receptor (PPAR)-γ knockdown mice (2). In the present study, we found that relatively short-term administration of a nonatherogenic HFD impaired the ability of smaller arterioles to dilate and altered the contractile and dilatory properties of basilar arteries only after ischemic injury. Interestingly, these detrimental changes in cerebrovascular function were in the absence of overt obesity. There is no definition of obesity in animal models such as it is clearly defined in humans as body mass index (BMI) > 30. A person has traditionally been considered to be obese if he or she is >20% over ideal weight. In our animals total body weight increased by 10%, and this was mainly adipose mass. Thus the changes we observed in this model are mainly the effect of HFD and not obesity per se.

Cerebral vascular function is closely regulated by central nervous system activity, especially astrocytes whose processes are in direct contact with both synapses and blood vessels (20). Previous reports demonstrated the contribution of astrocytes in neurovascular coupling through K⁺ signaling (3, 9). In the present study, we evaluated whether K⁺-mediated vasodilation is disrupted after HFD treatment. We found that whisker stimulation-induced functional hyperemia (in vivo) and K⁺-induced vasodilation (in vitro) are reduced in the HFD group. The data suggest that PAs from the HFD group had impaired vascular function. Given the lack of increased blood flow

![Image](http://ajpregu.physiology.org/)

**Fig. 4.** Effect of HFD on neuronal injury in different models of cerebral ischemia. Focal ischemia (A) induced by 3-h MCAO and 21-h reperfusion increased infarct size in the HFD group, but 10-min global ischemia (B) did not impact neuronal death in the hippocampus (n = 10–18/group). Values are medians in A and means ± SE in B. *P < 0.05.

![Image](http://ajpregu.physiology.org/)

**Fig. 5.** Effect of HFD on vascular function after focal cerebral ischemia-reperfusion. The balanced edema percentage (A) was not significantly higher, but the occurrence rate (C) and the severity (B) of hemorrhagic transformation (HT) determined by excess Hb in the brain were greater in the HFD group (n = 18/group). Values are medians in A and means ± SE in B. *P < 0.05.
response following whisker stimulation, future studies addressing the role of astrocytes in activity-dependent vascular responses are needed to better define whether HFD only affected vascular function or if it also altered the activity of upstream mechanisms such as that of K+ signaling by astrocytes.

In the present study we found no significant effect of HFD on surviving neuronal cell number in the GCI model even with different methods (NeuN staining, data not shown), which is consistent with another report that used 60-day Western HFD in Sprague-Dawley rats and also found no effect of HFD on neuronal cell death or survival after GCI (1). While we did not assess functional end points after GCI in our study, the study by Arvanitidis et al. (1) did assess functional outcome with the Morris water maze and found no significant effect of HFD, a finding consistent with the lack of effect of HFD on neuronal cell death or survival in both our and their studies. In a preliminary study, we utilized an even longer HFD period of 10 wk, with the thought that a longer duration may be needed to observe an effect in the GCI model. However, 10-wk HFD also had no significant effect on neuronal cell death/survival after GCI. It is not clear as to why HFD increased neuronal damage in the FCI but not GCI model of cerebral ischemia. It is known that the pathophysiological mechanisms differ between the two models (e.g., a more delayed neuronal cell death occurring in vulnerable brain regions after GCI), which might contribute to the difference. In addition, the duration of ischemia is also quite different between the two models (3 h in FCI vs. 10 min in GCI), which might contribute to differences in effects. While the mechanisms underlying the differential effect of HFD on neuronal cell death/survival in the two ischemia models requires further study, the significant effect of HFD observed in the FCI model is of potential translational importance. This is especially true considering that, of the two ischemia models, the FCI (MCAO) model is generally accepted as the most translationally relevant model of ischemic stroke, as >75% of strokes in humans involve occlusion of the MCA.

Obesity is an independent risk factor and may affect other risk factors for stroke such as hypertension, diabetes, and hyperlipidemia. Experimental studies have shown that either HFD or genetically induced obesity was accompanied by increased cerebrovascular remodeling, promoted hypertension, and increased infarct size in either transient or permanent focal ischemia models (7, 32). HFD-fed apoE−/− mice with hyperlipidemia also had increased infarct volume (23). However, another report showed that 1-mo HFD had no effect on the cerebral ischemia outcome (26). In the present study, 8-wk HFD resulted in significantly larger infarct volume after transient focal ischemia induced by suture occlusion of MCA, which is comparable to previous reports. When a global ischemia model was employed, there was no difference in hippocampal neuronal death between the groups, which was also reported by another group (1). These findings suggest that the duration of the diet and the method of ischemia are important for the extent and localization of neuronal injury. While we do not know the potential mechanisms contributing to greater neurovascular injury and poor outcomes in our model, it is possible that proper regulation of cerebral perfusion after stroke contributes to unfavorable outcomes. Since large arteries like the basilar artery can contribute significantly to total cerebrovascular resistance and are major determinants of microvascular pressure, dysregulation of basilar artery function may worsen stroke injury by altering cerebral perfusion after stroke. In this context, it is highly possible that exacerbated release of vasoactive factors, such as ET-1, released into the circulation may be mediating this response. In a recent elegant study from Dr. Cipolla’s group, investigators showed that plasma from hyperglycemic animals can affect cerebrovascular function through peroxynitrite generation and ET-1 (34). In another study, we showed that stroke decreases the dilatory ability of basilar arteries in regular chow-fed animals compared with sham treatment (6), and administration of atenolol, an ET receptor antagonist, at reperfusion prevented this response. While the experimental conditions of that particular study were different, maximum relaxation observed in sham-treated rats (~50%) was reduced to ~25% and this was normalized by ET receptor antagonism. In the present study, we do not have a sham treatment group, but it is possible that even CD-fed animals may be displaying some degree of dysfunction and this is exacerbated in HFD. We have previously shown that HFD increases plasma ET-1 (38). Given these findings, the ET system may play a role in exacerbated stroke injury in our model and will be further pursued.

Along the same lines, in light of our recent studies showing that augmented O-GlcNAcylation increases vascular reactivity to ET-1 (28), we next assessed whether this posttranslational modification is a potential downstream mechanism contributing to HFD-induced vascular dysfunction. As recently reviewed, there are multiple targets that are regulated by
O-GlcNAcylation in the vasculature (30). A positive correlation between phosphorylation of the MAPK cascade (ERK1/2 and p38) and nuclear O-GlcNAcylation was observed in fetal human cardiac myocytes exposed to high glucose (14). Previous work from our group has shown that O-GlcNAcylation-induced increased reactivity of aorta to PE was prevented by a PKC inhibitor or a Rho kinase inhibitor, respectively (17, 29). Our present finding of increased O-GlcNAc levels in basilar arteries of HFD-fed animals after stroke merits further studies to determine the mechanisms linking HFD to increased O-GlcNAcylation as well as linking O-GlcNAcylation to cerebrovascular dysfunction. In a preliminary study, we found that HFD alone caused a small increase (1.5 fold) in O-GlcNAc levels compared with a fourfold increase observed in this study with HFD + MCAO. It is of great interest to determine whether blockade of increased O-GlcNAc levels prevents vascular dysfunction and improves stroke outcomes.

Perspectives and Significance

In the present study, the important findings of impaired neurovascular communication, large-artery dysfunction, and augmented neurovascular injury suggest that even short-term HFD without obesity or metabolic imbalance may be detrimental to the cerebrovasculature and exacerbate the response to cerebral ischemia. We recognize that there are limitations to this study such as evaluation of the outcome only at 24 h and inclusion of only male rats. Given that AIS has dramatically increased in children and young adults, which is positively correlated with increases in risk factors including obesity, lipid disorders, and diabetes (13), further studies are warranted to explore the underlying mechanisms by which HFD worsens short- and long-term stroke outcome in both female and male animal models.

GRANTS

A. Ergul is a research pharmacologist at the Charlie Norwood Department of Veterans Affairs (VA) Medical Center. This work was supported in part by VA Merit Award BX00347 to A. Ergul), a Georgia Health Sciences University Diabetes and Obesity Discovery Institute Synergy Award (S. P. Didion, J. A. Filosa, D. W. Brann, R. C. Tostes, and A. Ergul), an American Heart Association Established Investigator Award (0740002N to A. Ergul), and National Institutes of Health Grants HL-089884 and HL-107632 to S. P. Didion, HL-089067 to J. A. Filosa, NS-050730-08 to D. W. Brann, and NS-054688 to A. Ergul.

DISCLOSURES

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

AUTHOR CONTRIBUTIONS

Author contributions: W.L., R.P., D.C., W.D., Q.Z., and V.V.L. performed experiments; W.L., R.P., D.C., W.D., Q.Z., and V.V.L. analyzed data; W.L., R.C.T., and A.E. interpreted results of experiments; W.L. and V.V.L. prepared figures; W.L., R.P., J.A.F., and D.W.B. drafted manuscript; W.L., R.C.T., and A.E. edited and revised manuscript; S.F.D., J.A.F., D.W.B., R.C.T., and A.E. conceived and designed of research; S.P.D., J.A.F., D.W.B., and A.E. approved final version of manuscript.

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