Nox2-Derived Superoxide Contributes to Cerebral Vascular Dysfunction in Diet-Induced Obesity

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Background and Purpose—Obesity is an increasing epidemic worldwide; however, little is known about effects of obesity produced by high-fat diet (HFD) on the cerebral circulation. The purpose of this study was to examine the functional and temporal effects of a HFD on carotid and cerebral vascular function and to identify mechanisms that contribute to such functional alterations.

Methods—Responses of cerebral arterioles (in vivo) and carotid arteries (in vitro) were examined in C57Bl/6 (wild-type) and Nox2-deficient (Nox2−/−) mice fed a control (10%) or a HFD (45% or 60% kcal of fat) for 8, 12, 30, or 36 weeks.

Results—In wild-type mice, a HFD produced obesity and endothelial dysfunction by 12 and 36 weeks in cerebral arterioles and carotid arteries, respectively. Endothelial function could be significantly improved with Tempol (a superoxide scavenger) treatment in wild-type mice fed a HFD. Despite producing a similar degree of obesity in both wild-type and Nox2−/− mice, endothelial dysfunction was observed only in wild-type, but not in Nox2−/−, mice fed a HFD.

Conclusions—Endothelial dysfunction produced by a HFD occurs in a temporal manner and appears much earlier in cerebral arterioles than in carotid arteries. Genetic studies revealed that Nox2-derived superoxide plays a major role in endothelial dysfunction produced by a HFD. Such functional changes may serve to predispose blood vessels to reduced vasodilator responses and thus may contribute to alterations in cerebral blood flow associated with obesity. (Stroke. 2013;44:00-00.)

Key Words: brain ■ diabetes mellitus, type 2 ■ diet, high-fat ■ oxidative stress

Obesity, currently a leading public health concern, is associated with an increased risk of vascular disease and cardiovascular events, including carotid artery disease and stroke.1−3 A diet high in caloric fat content is a major contributor to the increasing global incidence of obesity. Previous studies have shown that obesity is associated with endothelial dysfunction in peripheral and cerebral blood vessels.4−12 Although much of the information about the effects of obesity on the cerebral circulation has come from genetic models of obesity (such as leptin or leptin receptor deficiency),10−12 little is known about the effects of obesity produced by a high-fat diet (HFD) on cerebral blood vessels.

In obese humans, there is a positive correlation between visceral adiposity and markers of oxidative stress.5,9,13,14 Increases in oxidative stress, particularly superoxide, serve to limit nitric oxide bioavailability and in turn endothelial function.15 An important source of vascular superoxide is derived from the enzymatic activity of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase.16−19 Although expression of NADPH oxidase seems to be increased in adipose and blood vessels in obesity,20,21 the specific contribution of Nox2 (an important catalytic component of NADPH oxidase) to the impairment of endothelial function in diet-induced obesity has not been defined.

Considering that the incidence of obesity is increasing worldwide and that obesity has been independently linked to cognitive decline,22−24 the first goal of the present study was to examine the temporal and functional effects of HFD on endothelial responses in the carotid artery and cerebral microcirculation. The second goal was to examine the specific contribution of Nox2 to the development of obesity and alterations in cerebral vascular function produced by a HFD.

Methods

Experimental Animals

Male C57Bl/6 (wild-type; No. 000664) and homozygous Nox2-deficient (Nox2−/−) (B6.129S6-Cybbtm1Din/J; No. 002365)25 4- to 8-week old mice were obtained from Jackson Laboratories (Bar Harbor, Maine).

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Harbor, ME). All protocols conformed to the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee.

**Experimental Protocols**

Wild-type mice were fed either a control diet (10% kcal of fat, No. D12450B from Research Diets, New Brunswick, NJ) or a HFD (45% or 60% kcal of fat, No. D12451 and No. D12492, respectively) for 8, 12, 30, or 36 weeks. Nα2−/− mice were fed either a control or 45% HFD for 12 weeks (cerebral arteriole studies) or a control or 45% or 60% HFD for 36 weeks (carotid studies).

**Studies of Cerebral Arteriolar and Carotid Artery Function**

Methods used to examine responses of cerebral arterioles and carotid arteries in the present study have been described previously by our laboratory and are described in detail in the online-only Data Supplement.

**Statistical Analysis**

All data are expressed as mean±SE. Cerebral responses are presented as a percentage change in diameter compared with baseline, and statistical analyses are performed using paired or unpaired *t* tests. Responses of carotid artery are expressed as a percentage relaxation to U46619-induced contraction. Comparisons of relaxation and contraction were made using ANOVA followed by Bonferroni multiple comparisons test. Statistical significance was accepted with *P*<0.05.

**Results**

**Phenotypic Assessment of Obesity Development**

In wild-type mice fed a 45% HFD for 8 to 36 weeks, body weight and perirenal fat mass were significantly greater (*P*<0.05) than that in wild-type mice fed a control diet (Figure 1). These same parameters were also greater, but to a significantly larger extent (*P*<0.05), in wild-type mice fed a 60% HFD compared with either control or 45% HFD fed wild-type mice (Figure 1). Both HFDs produced hyperglycemia in wild-type mice; however, the degree of hyperglycemia produced by the 2 diets was similar (*P*<0.05; Figure 1).

**Effect of a HFD on Responses of Cerebral Arterioles**

Acetylcholine produced dilatation of cerebral arterioles in wild-type mice fed a control diet, and this response was not affected (*P*>0.05) by the length of time (8, 12, or 30 weeks) on the diet (Figure 2). After 8 weeks of either a 45% or 60% HFD, responses to acetylcholine were similar to that observed in wild-type mice fed a control diet for 8 weeks (Figure 2). In contrast, responses of cerebral arterioles to acetylcholine were markedly reduced in mice fed a 45% HFD for 12 and 30 weeks (Figure 2). We anticipated that a 60% HFD might impair responses to acetylcholine at earlier time points and to a greater extent. However, the degree of dysfunction produced by a 60% HFD was similar to that produced by a 45% HFD at 12 weeks. Because a 60% HFD did not produce any additional degree of impairment, we elected not to extend studies with the 60% HFD to 30 weeks. Endothelium-independent responses to nitroprusside in cerebral arterioles were not altered by diet or time on diet (Figure I in the online-only Data Supplement).

**Effect of a HFD on Responses of Carotid Arteries**

Responses to acetylcholine were similar in carotid arteries from mice fed a 45% HFD for 8, 12, or 30 weeks compared with mice fed a control diet (Figure II in the online-only Data Supplement and Figure 2B). In contrast, responses of carotid arteries to acetylcholine were markedly impaired in mice fed a 45% HFD for 36 weeks compared with mice fed a control diet for the same length of time (Figure 2). Endothelium-independent responses to nitroprusside in carotid artery were unaffected by either HFD or length of time on each diet (Figure III in the online-only Data Supplement).

**Endothelial Dysfunction Produced by a HFD Is Mediated by Superoxide**

Because both a 45% and 60% HFD produced similar degrees of impairment in the cerebral circulation, we elected to examine mechanisms in response to a 45% HFD for 12 weeks in the cerebral circulation and for 36 weeks in carotid arteries in subsequent studies. To address a role for superoxide, vascular responses in carotid artery or cerebral arterioles were examined in the presence or absence of Tempol, a superoxide scavenger. Although Tempol had no effect on baseline cerebral arteriolar
diameter (Figure IV in the online-only Data Supplement), acetylcholine-induced vasodilation was significantly improved in Tempol-treated vessels (Figure V in the online-only Data Supplement). Similarly, responses of carotid arteries to acetylcholine in 36 weeks HFD mice could be improved after Tempol-treatment. In separate experiments, vascular superoxide levels (as measured using electron spin resonance) in aortic homogenates were significantly higher in wild-type mice fed a HFD compared with wild-type mice fed a control diet (Figure VI in the online-only Data Supplement).

Development of Obesity Produced by a HFD Is Independent of Nox2
Body weight and visceral adiposity were found to be similar to that in wild-type mice fed a control diet for 12 weeks or 36 weeks; however, glucose levels were found to be lower in Nox2−/− fed a control diet compared with wild-type mice (Figures 3A and 4A). Both 45% and 60% HFDs produced significant, but similar, increases in body weight, visceral adiposity, and hyperglycemia in wild-type and Nox2−/− mice (Figures 3A and 4A).

Because a HFD may also promote insulin resistance and because measures of plasma glucose alone may not be reflective of insulin resistance per se, we assessed insulin sensitivity by performing glucose tolerance tests in wild-type and Nox2−/− mice under baseline conditions (before the start of the experimental diets) and in wild-type and Nox2−/− mice fed a control or HFD for 12 weeks. Under baseline conditions, wild-type and Nox2−/− mice were able to clear a glucose bolus (1 g/kg IP) within 120 minutes, suggesting that deficiency of Nox2 is not associated with alterations in insulin sensitivity. In contrast, 12 weeks of a HFD was associated with insulin resistance, as evidenced by a much slower rate of glucose clearance. Perhaps more importantly, deficiency of Nox2 prevented the impairment of insulin sensitivity because Nox2−/− mice were able to clear the glucose bolus at a similar rate as wild-type mice fed a control diet (Figure 5). Taken together, these data suggest that although the development of obesity per se is not affected by Nox2 deficiency, it seems that Nox2-derived superoxide contributes to the development of insulin resistance.

Endothelial Dysfunction Produced by a HFD Is Mediated by Nox2
In an attempt to identify the enzymatic source of superoxide responsible for the impairment of endothelial function, we next performed experiments to determine the role of NADPH oxidase in the impairment of vascular function in our model.

Figure 2. A, Dilatation of cerebral arterioles to acetylcholine was markedly impaired in wild-type mice fed a 45% high-fat diet (HFD) for 12 and 30 weeks, but not 8 weeks compared with those in wild-type mice fed a control diet. A 60% HFD produced a similar degree of impairment of cerebral arteriolar function in wild-type mice, which also occurred in a temporal manner. B, A 45% HFD for 36 weeks, but not 12 or 30 weeks, was associated with a marked impairment of acetylcholine-induced relaxation in carotid artery compared with wild-type mice fed a control diet. Tempol restored endothelial responses in wild-type mice fed a 45% HFD for 36 weeks, suggesting that the impairment of endothelial function was mediated by superoxide. Means±SE. *P<0.05 vs control.

Figure 3. A, Body weight gain, perirenal adipose mass, and fasting blood glucose levels in wild-type and Nox2-deficient (Nox2−/−) mice fed either a control or 45% high-fat diet (HFD) for 12 weeks. B and C, Although a 45% HFD was associated with marked impairment of acetylcholine-induced dilatation in wild-type mice, cerebral arteriolar response in Nox2−/− mice was similar to that in wild-type mice fed a control diet and was not affected by a 45% HFD. Means±SE. *P<0.05 vs respective control. †P<0.05 vs wild type; n= 6 to 10.
Vascular Nox2 expression was higher in aorta from wild-type mice fed a 45% HFD compared with their control diet counterparts (Figure 6), consistent with our measurements of vascular superoxide (Figure VI in the online-only Data Supplement).

In separate experiments, we also sought to determine a potential mechanism that might contribute to the impairment of endothelial function in cerebral arterioles, but not carotid artery, after a 12-week HFD. Thus, we elected to examine the expression of endothelial nitric oxide synthase (eNOS) and CD36 because endothelium-dependent response in cerebral arterioles have been shown to be mediated, in large part, by nitric oxide and eNOS and as CD36 (a major scavenger receptor responsible for the uptake of oxidized low-density lipoprotein [LDL]) has been shown previously to be associated with endothelial dysfunction. Expression of eNOS and CD36 was found to be similar in brains from wild-type mice fed a control or HFD (Figure IX in the online-only Data Supplement). Taken together, our molecular data demonstrate that a HFD is associated with increased vascular expression of Nox2 and independent of alterations in eNOS and CD36 expression. Although these molecular findings provide additional supporting evidence that the impairment of endothelial function in obesity is mediated, in large part, by increases in Nox2-derived superoxide, we cannot completely rule out the possibility that translational and post-translational alterations in eNOS and CD36 may also play a role. For example, CD36...
expression has been shown to be modulated at the level of translation under conditions of hyperglycemia.30

Endothelial responses were normal in Nox2−/− mice fed a control diet. In contrast, a 45% and 60% HFD produced marked impairment of endothelial responses in wild-type mice, whereas a HFD did not produce alterations in endothelial responses in either cerebral arterioles or carotid arteries of Nox2−/− mice (Figures 3B and 4B). Endothelium-independent responses to nitroprusside were not altered by Nox2 deficiency alone or Nox2 deficiency in combination with a HFD in either cerebral arterioles (data not shown) or carotid arteries (Figure 4B and 4C).

Discussion
There are several major new findings of the present study. First, a HFD produced endothelial dysfunction that occurred in a temporal manner and appeared much earlier in cerebral arterioles than that in carotid arteries. Second, endothelial dysfunction produced by high fat was mediated by superoxide because a superoxide scavenger was effective at improving endothelial function in wild-type mice fed a HFD. Third, endothelial dysfunction in both carotid arteries as well as the cerebral circulation was absent in Nox2-deficient mice fed a HFD. Taken together, these findings provide the first direct evidence that Nox2-derived superoxide contributes to carotid and cerebral endothelial dysfunction in a model of diet-induced obesity.

Obesity Is Associated With Endothelial Dysfunction in the Cerebral Circulation
Endothelium-dependent responses in cerebral blood vessels are impaired in many genetic models of obesity, mostly models of leptin and leptin receptor deficiency.11,12,27,28 Although such genetic models are informative, deficiency of either leptin or the leptin receptor is extremely rare in humans and not always associated with obesity.30 In contrast, high-fat feeding is a well-established model of diet-induced obesity that recapitulates key features of obesity in humans, for example, mice fed a HFD primarily gain weight in the form of visceral adipose (intra-abdominal fat) similar to that in obese humans.32–34 In the present study, a HFD produced a marked degree of obesity as evidenced by increased visceral adiposity in wild-type mice. In addition, the HFD produced hyperglycemia because fasting glucose levels were significantly higher in high-fat–fed wild-type mice. These findings are consistent with previous studies using the same (or similar) HFD to produce obesity and hyperglycemia in C57Bl/6 mice.32–36

Although obesity is associated with endothelial dysfunction in many peripheral blood vessels, little is known about the effect of diet-induced obesity on endothelial responses in cerebral blood vessels and even less is known on temporal effects.4–12 In the present study, we examined the temporal effects of a HFD on responses in carotid artery and cerebral blood vessels. We found that a HFD was associated with temporal increases in body weight and adiposity in wild-type mice. However, despite marked increases in adiposity early on, endothelium-dependent responses to acetylcholine were similar in cerebral arterioles of wild-type mice fed a HFD for 8 weeks compared with wild-type mice fed a control diet. By 12 weeks, cerebral arteriolar responses to acetylcholine were impaired (=50% less than that observed in wild-type mice fed a control diet) in wild-type mice fed a HFD. Arteriolar responses to acetylcholine were impaired to a similar extent in wild-type mice fed a 45% HFD for 30 weeks as that observed at 12 weeks. These data suggest that a HFD impairs endothelial function in cerebral arterioles by ≥12 weeks and that a HFD is not associated with additional reductions in endothelial responses in cerebral arterioles with longer time points of high-fat feeding, at least ≤30 weeks.

In contrast, responses to acetylcholine were normal in carotid arteries from wild-type mice fed either a control or 45% HFD for 8 to 30 weeks. Endothelial dysfunction eventually became evident in carotid arteries in wild-type mice but only after 36 weeks of high-fat feeding. These data suggest that unlike cerebral arterioles, where endothelial dysfunction is apparent as early as 12 weeks, a much longer exposure to a HFD is required to produce similar dysfunction in carotid arteries. These data suggest that cerebral arterioles seem to be more sensitive than carotid artery to the effects of a HFD. Although the specific mechanism(s) that account for the earlier impairment of endothelial function produced by a HFD are unclear, it is possible that these effects may be reflective of the fact that cerebral blood vessels have been shown to have a higher capacity to generate superoxide. Indeed, it has been shown previously that NADPH oxidase activity and function are greater in cerebral versus that in systemic arteries.37 The fact that Tempol could improve endothelial function in both cerebral arterioles and carotid arteries in wild-type mice fed a HFD and that Nox2 deficiency served to protect endothelial function would also be consistent with this concept.

In addition to the temporal effects of a HFD on vascular responses, little is known about the effect of varying the percentage of dietary fat on vascular responses. Thus, we compared and contrasted the effects of a 45% HFD with that of a 60% HFD. We predicted that the degree of impairment would be directly proportional to the caloric fat content present in the 2 diets. However, although a 60% HFD produced a greater degree of obesity and visceral adiposity, it was not associated with a greater degree of endothelial dysfunction in either cerebral arterioles or carotid arteries than that produced by a 45% HFD. Although the reason for this difference is not clear, we speculate that the increase in vascular Nox2 expression produced by a HFD is maximal with a 45% HFD and that increase in the fat content to 60% is simply not additive at the time points examined in the present study. The fact that Nox2 deficiency was sufficient to protect against the negative effects of a HFD on endothelial function is supportive in this possibility.

The effects of a HFD seemed to be selective for endothelium because responses to nitroprusside in both cerebral arterioles and carotid arteries were similar in HFD (both 45% and 60%) mice compared with that observed in control mice. Thus, the present findings demonstrate that endothelial dysfunction occurs in a temporal- and vessel-dependent manner and that the maximal degree of endothelial dysfunction produced by a HFD seems to occur with a 45% HFD. Taken together, these findings also established time points that then
allowed the design of studies to test mechanisms associated with impairment of endothelial function.

Endothelium-dependent responses in cerebral arterioles and carotid arteries in the mouse are mediated in large part by nitric oxide synthase and nitric oxide. It is fairly well established that increased superoxide contributes to endothelial dysfunction via superoxide-mediated inactivation of nitric oxide bioavailability. In the present study, a superoxide scavenger effectively restored cerebral arteriolar and carotid artery responses in wild-type mice fed a 45% or 60% HFD toward normal. In addition, vascular superoxide levels were found to be greater in wild-type mice fed a 60% HFD for 36 weeks compared with controls. Thus, our pharmacological data combined with measurements of superoxide strongly implicate a role for superoxide in the impairment of endothelial function in carotid and cerebral vessels in obesity.

**Nox2 Deficiency Limits Endothelial Dysfunction in the Cerebral Circulation Associated With a HFD**

Although our data with Tempol implicate superoxide in the impairment of endothelial function in the cerebral circulation produced by a HFD, it does not provide information about the enzymatic source of superoxide. Previous studies have implicated several sources of superoxide, such as xanthine oxidase, uncoupled eNOS, or NADPH oxidase, that contribute to endothelial dysfunction in many diseases that affect the vasculature such as diabetes mellitus and hypertension. Thus, to determine the enzymatic source of superoxide in our model, we examined obesity development and vascular function after a HFD in mice genetically deficient in Nox2, a major catalytic isofrom of NADPH oxidase.

Body weight, perirenal adipose mass, as well as plasma glucose levels were similar in wild-type and Nox2−/− mice fed a control diet. A HFD produced similar increases in body weight and perirenal adipose mass, as well as plasma glucose levels, in wild-type and Nox2−/− mice. Taken together, these data suggest that Nox2 deficiency does not alter metabolic phenotypes under baseline conditions or in response to a HFD. In terms of vascular responses, Nox2 deficiency was not associated with alterations in endothelial function because responses to acetylcholine were similar in wild-type and Nox2−/− mice fed a control diet. These data are consistent with previous findings and suggest that normally superoxide levels are low and that under normal conditions Nox2 expression and activity is not sufficient to limit endothelial function.

Although Nox2 deficiency did not affect obesity development, endothelial responses in both cerebral arterioles and carotid arteries to acetylcholine were normal in Nox2−/− mice fed a HFD. There are many comorbidities associated with a HFD that may ultimately serve to promote increases in vascular superoxide and endothelial dysfunction, it is often difficult to establish cause and effect relationships. Although hypercholesterolemia might be considered to be an important factor in this regard, previous studies have shown that a HFD is associated with only minimal to modest increases in plasma cholesterol in wild-type (C57Bl/6) mice. This is in contrast to the marked increase in plasma cholesterol levels observed in atherosclerotic mouse models, such as the apolipoprotein E- or the LDL receptor-deficient mice fed a normal (500–800 mg/dL range) or Western (high cholesterol; >2000 mg/dL) diet. More importantly, endothelial function seems to be normal in wild-type, apolipoprotein E, and LDL receptor mice in which plasma cholesterol levels are elevated as high as 500 mg/dL while on a normal diet. In contrast, a high cholesterol diet seems to impair endothelial function in apolipoprotein E and LDL receptor–deficient mice, but not in wild-type mice.

Similarly, many studies have shown that a HFD is associated with minimal to modest alterations in plasma LDL, high-density lipoprotein, and triglyceride levels. Based on such previous studies, we would predict that the HFD would have minimal effects on plasma lipids in the wild-type (C57Bl/6) mice used in the present study. Based on a recent report, we would also predict that Nox2 deficiency per se or in combination with a HFD would have little to no effect on plasma lipids. Thus, although it may be difficult to assign a specific role for any one factor in promoting the endothelial dysfunction observed with a HFD, our data in Nox2-derived superoxide in the impairment of endothelial function in carotid artery and cerebral microvessels is most likely related to the effects of a HFD independent of alterations in plasma lipid profiles.

**Conclusions**

These findings provide genetic evidence for a major role of Nox2-derived superoxide in impairment of endothelial function in carotid artery and cerebral microvessels in response to a HFD. Our data also demonstrate that Nox2 deficiency is not associated with alterations in the development of obesity produced by high fat, suggesting that the protective effect of Nox2 deficiency on endothelial function may reflect loss of Nox2 in the vessel wall. Although results derived from the present study cannot differentiate between the effects of obesity and hyperglycemia per se on endothelial function, we would propose that impairment of vascular function in carotid and cerebral microvessels could conceivably contribute, in part, to the cognitive decline and dementia associated with obesity and type II diabetes mellitus. This idea is consistent with emerging data that suggest that obesity with a high degree of visceral adipose accumulation is associated with an increased risk of dementia.

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**Disclosures**

None.

**References**


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Supplemental Methods.

**Studies of Cerebral Arteriolar Function.** Methods used to measure responses of cerebral (pial) arterioles (in vivo) have been described previously.\(^1\)\(^2\) Briefly, a cranial window was made over the parietal cortex and a segment of a cerebral arteriole was exposed for measurement of vascular diameter. All drugs were applied topically over the cranial window. Diameter of a single arteriole per animal was measured under control conditions and during topical application of drugs. Baseline cerebral arteriolar diameter was similar in all groups of mice and averaged 31±1 microns in diameter. Changes in diameter were measured in response to the endothelium-dependent dilator, acetylcholine (1 and 10 µmol/L) and to the endothelium-independent dilator nitroprusside (0.1 and 1 µmol/L). In some experiments, responses to acetylcholine and nitroprusside were determined and then repeated in the presence of vehicle (saline) or Tempol (1 mM). Arterial pressure under anesthesia was similar in all groups and averaged ~70 mmHg. Arterial blood gasses were measured and averaged pCO\(_2\), 32±1mmHg; pO\(_2\), 160±5mmHg; and pH, 7.32±0.01 (Mean±SE). At the conclusion of each experiment, samples of arterial blood were saved for determinations of blood glucose (fasting) using an Accu-Chek Advantage glucometer (Roche; Indianapolis, IN).

**Studies of Carotid Artery Function.** Carotid artery responses (in vitro) were examined in isolated organ chambers as described previously.\(^3\)\(^4\) Following a 45-minute equilibration period, vessels were precontracted (50-60% of maximum) with the thromboxane analogue, 9,11-dideoxy-11a, 9a-epoxymethanoprostaglandin F\(_{2\alpha}\) (U46619). After reaching a stable contraction plateau, concentration-response curves were generated to acetylcholine (0.01 to 100 µmol/L) and nitroprusside (0.03 to 30 µmol/L). In some experiments, carotid
arteries were incubated with either vehicle or 4-hydroxy-2,2,6,6-
tetramethylpiperidine 1-oxyl (Tempol, 1 mM; a superoxide scavenger).

**Glucose Tolerance Test.** Glucose tolerance tests were performed in wild-type and *Nox2*−/− under baseline conditions (ie, before placement on a control or HFD) and in wild-type and *Nox2*−/− mice fed either a control or HFD for 12 wks as described previously.5 Briefly, mice were fasted overnight (16 hrs). Baseline glucose levels (fasting) were measured in each mouse followed by a bolus intraperitoneal glucose (1 g/kg) injection. Glucose levels were then determined at 15, 30, 60, 90 and 120 min post-glucose injection.

**Quantitative real-time RT-PCR.** Gene expression was analyzed in wild-type mice fed either a control or HFD for 12 wks using quantitative real-time RT-PCR as previously described.6 RNA from whole brain was extracted using TRlzol reagent (Invitrogen, Carlsbad, CA) and purified using Mini RNeasy kit (Qiagen, Valencia, CA). cDNA was obtained by reverse transcription and gene expression for endothelial nitric oxide synthase and CD36 were evaluated using SYBR-green dye chemistry on a Bio-Rad CFX96 platform.

**Drugs and Chemicals.** Acetylcholine, nitroprusside, and Tempol were obtained from Sigma (St. Louis, MO) and all were dissolved with saline. U46619 was obtained from Cayman Chemical (Ann Arbor, MI) in peanut oil and dissolved in 100% ethanol with all subsequent dilutions being made with saline. All other reagents were of standard laboratory grade.
Supplemental Figures and Figure Legends.

Supplemental Figure I. Endothelium-independent responses to nitroprusside were similar in cerebral arterioles from wild-type mice fed a control diet or a 45% HFD for 8, 12, and 30 wks. These findings suggest that the impairment of endothelium-dependent responses to acetylcholine produced by a HFD was selective for endothelium. Mean±SE; 8 wks, n=6; 12 wks, n=4-6; 30 wks, n=6; P>0.05.
Supplemental Figure II. Responses to acetylcholine and nitroprusside were similar in carotid arteries from wild-type mice fed either a control diet or 45% HFD for 8 wks, suggesting that this diet and duration was not associated with impairment of function in the carotid artery. Mean±SE; n=3/group; P>0.05.
Supplemental Figure III. Responses to nitroprusside were similar in carotid arteries from wild-type mice fed either a control or a 45% HFD for 12-36 wks. These findings indicate that the effect of a 45% HFD on responses to acetylcholine in the carotid artery were selective for endothelium. Mean±SE; P>0.05.
Supplemental Figure IV. Tempol had no effect on baseline diameter in cerebral arterioles of wild-type mice fed either a control or a 45% HFD for 12 wks. Arteriolar diameter was measured before (vehicle) and then re-measured following 30 min suffusion of the cranial window with Tempol (1 mM). Mean±SE; n=5/group; P>0.05.
Supplemental Figure V. Dilatation produced by acetylcholine was similar in cerebral arterioles following suffusion with vehicle or Tempol in wild-type mice fed a control diet for 12 wks. Dilatation in response to acetylcholine was significantly improved in cerebral arterioles from wild-type mice fed a 45% HFD for 12 wks treated acutely with Tempol. These findings implicate a role for superoxide in the impairment of endothelial function produced by a HFD. Mean±SE; n=5/group; *P<0.05 vs. vehicle.
Supplemental Figure VI. A) Representative ESR spectra from vascular (aortic) homogenates from wild-type mice fed a control or a 60% HFD for 36 wks. Superoxide levels were measured using the spin probe 1-hydroxy-3-methoxycarbonyl-2,2,5,5,-tetramethylpyrrolidine•HCl (CMH; Alexis Biochemicals, San Diego, CA). Aortic homogenates (1 mg/ml) from each experimental group were incubated with 75 µl of spin-trap stock solution consisting of CMH (20 µM in DPBS plus 25 µM desferrioxamine). ESR spectra were measured using a benchtop ESR (Magnettech) at a microwave power of 40 mW, modulation amplitude of 3,000 mG and modulation frequency of 100 kHz. The ESR signal was significantly attenuated in the presence of PEG-SOD (100 U/ml) in vascular homogenates from mice fed a HFD providing confirmation that the signal was due to superoxide. B) The peak ESR amplitude was significantly greater in wild-type mice fed a HFD (n=4) as compared to wild-type mice fed a control diet (n=3). Mean±SE; *P<0.05 vs. control.
Supplemental Figure VII. Endothelial nitric oxide synthase (eNOS) and CD36 expression as assessed by real-time RT-PCR in whole brain from wild-type mice fed either a control (CNT) diet or HFD for 12 wks. Mean±SE; n=5/group; P>0.05.
Supplemental References.


