Inflammatory and Injury Responses to Ischemic Stroke in Obese Mice

Satoshi Terao, MD; Gokhan Yilmaz, MD; Karen Y. Stokes, PhD; Mami Ishikawa, MD, PhD; Takeshi Kawase, MD, PhD; D. Neil Granger, PhD

Background and Purpose—Although epidemiological studies reveal an increased incidence of obesity and an association between obesity and the prevalence/severity of ischemic stroke, little is known about the mechanisms that link obesity to ischemic stroke. This study tested the hypothesis that obesity exacerbates the cerebrovascular dysfunction and tissue injury induced by brain ischemia and reperfusion.

Methods—The adhesion of leukocytes and platelets in cerebral venules, blood–brain barrier permeability, brain water content, and infarct volume were measured in wild-type, obese (ob/ob), and leptin-reconstituted ob/ob mice subjected to 30 minutes middle cerebral artery occlusion and reperfusion. Tissue and plasma cytokine levels were determined by cytometric bead array, and a role for monocyte chemoattractant protein-1 and interleukin-6 was assessed using blocking antibodies.

Results—Compared with wild-type mice, ob/ob exhibited larger increases in leukocyte and platelet adhesion, blood–brain barrier permeability, water content, and infarct volume after middle cerebral artery occlusion–reperfusion. Reconstitution of leptin in ob/ob mice tended to further enhance all reperfusion-induced responses. Ob/ob mice also exhibited higher plasma levels of monocyte chemoattractant protein-1 and interleukin-6 than wild-type mice. Immunoneutralization of monocyte chemoattractant protein-1, but not interleukin-6, reduced infarct volume in ob/ob mice.

Conclusions—Obesity worsens the inflammatory and injury responses to middle cerebral artery occlusion and reperfusion by a mechanism independent of leptin deficiency. Monocyte chemoattractant protein-1 appears to contribute to the exaggerated responses to ischemic stroke in obese mice. (Stroke. 2008;39:943-950.)

Key Words: cerebral infarct ■ chemokines ■ cytokines ■ obesity ■ platelets

The incidence of obesity in industrialized countries has increased abruptly and is now reaching epidemic proportions. It is estimated that over two-thirds of Americans are either overweight or obese. Obesity is a major healthcare problem because it increases the morbidity and mortality of a variety of diseases, including cardiovascular diseases, cancer, and sepsis.1 The impact of obesity on cardiovascular disease has earned it the designation of risk factor, along with hypertension, hypercholesterolemia, diabetes, and smoking. Epidemiological studies have revealed that obesity increases the risk for coronary artery disease.2 Similarly, there is evidence that obesity increases the risk of ischemic stroke.3-5 These studies also indicate that the increased risk for stroke in overweight or obese subjects is independent of diabetes, hypertension, or hypercholesterolemia.3 Because obesity is a modifiable risk factor, weight loss has the potential to prevent stroke.

There is some evidence indicating that obesity not only increases disease incidence, but also increases disease severity. For example, myocardial ischemia in obese patients is associated with larger infarctions than the normal population.6 Experimental studies have also revealed more severe inflammatory and tissue injury responses in obese mice. For example, obese mice exhibit more intense cerebral microvascular dysfunction, inflammation, and behavioral deficits during sepsis induced by cecal ligation and puncture compared with lean mice.7 Despite the epidemiological evidence linking obesity to ischemic tissue diseases, there has been little effort to determine if and how obesity influences disease severity in animal models of myocardial ischemia or stroke.

There is mounting evidence suggesting that obesity may exert its deleterious effects on the cardiovascular system by inducing an inflammatory state that targets both large and small blood vessels.5,9 A likely potential source of the mediators that induce the low-grade inflammation associated with obesity is adipose tissue. Adipose tissue is considered to be a highly active endocrine organ that liberates several cytokines and chemokines (collectively referred to as adipo-
kines) that can induce an inflammatory phenotype in distant tissues.\textsuperscript{10} This inflammatory mediator-releasing property of adipose tissue appears to account for the higher plasma levels of adipokines detected in clinically obese subjects.\textsuperscript{11,12} These observations, coupled with the well-established participation of inflammatory mechanisms in the pathogenesis of ischemic stroke, suggest that obesity may predispose the brain to exaggerated inflammatory and injury responses after ischemia–reperfusion (I/R). Hence, the overall objectives of the present study were to test the hypothesis that obesity exacerbates the microvascular dysfunction and brain damage induced by I/R and to assess the involvement of cytokines/chemokines in the exaggerated injury responses associated with obesity. Leptin-deficient \textit{ob/ob} mice were used to address these issues. Therefore, an additional effort was made to determine whether restoration of plasma leptin levels in \textit{ob/ob} mice altered the cerebral microvascular responses to I/R.

\textbf{Methods}

\textbf{Animal Preparation}

The experimental procedures used in this study were reviewed and approved by the Institutional Animal Care and Use Committee and were in compliance with the guidelines of the National Institutes of Health. Male C57BL/6/J mice (WT; \textit{N}=53), B6.V-Lep\textsuperscript{ob/ob} mice (\textit{ob/ob}; \textit{N}=62), and leptin-reconstituted B6.V-Lep\textsuperscript{+/+} mice (\textit{ob/+}; \textit{N}=40) were obtained from Jackson Laboratories (Bar Harbor, Maine). Young (5 to 7 week) \textit{ob/ob} mice were used to circumvent the potential influence of hyphoglycemia and hypercholesterolemia on the measured responses.\textsuperscript{7}

\textbf{Middle Cerebral Artery Occlusion and Reperfusion}

Mice were anesthetized with an intraperitoneal injection of ketamine (100 mg/kg) and xylazine (10 mg/kg). Transient (30 minutes) focal cerebral ischemia was induced by occlusion of the left middle cerebral artery (MCAO) using a modification of the intraluminal filament method\textsuperscript{13} with a 7-0 silicone-coated nylon monofilament (Doccol Corp). After the 30-minute occlusion period, the nylon fiber was gently removed and the common carotid artery was reopened. Ischemia and reperfusion were verified using a laser Doppler (Doccol Corp). After the 30-minute occlusion period, the nylon fiber was gently removed and the common carotid artery was reopened. Ischemia and reperfusion were verified using a laser Doppler (Doccol Corp). After the 30-minute occlusion period, the nylon fiber was gently removed and the common carotid artery was reopened. Ischemia and reperfusion were verified using a laser Doppler (Doccol Corp).

\textbf{Leptin Reconstitution Experiments}

At 4 hours after reperfusion, mice were anesthetized as stated previously. The cerebral microcirculation was visualized with an upright fluorescent microscope using a 20× water immersion lens. Color images were captured with a 3 charge coupled device (CCD) color video camera. Randomly selected segments of pial venules (25 to 50 \textmu m diameter, 100 \textmu m long) were chosen for observation. Approximately 100,000 platelets were isolated from a donor mouse, labeled ex vivo with carboxyfluorescein diacetate succinimidyl ester,\textsuperscript{13} and administered to recipient mice through the left femoral vein. Then 0.02% rhodamine 6G (which labeled [red] circulating leukocytes) was continuously infused. Adherent leukocytes and platelets were defined as cells remaining stationary within venules for \approx 30 seconds and 2 seconds, respectively. The number of adherent leukocytes with or without attached platelets was quantified as well as the number of platelets binding directly to venular endothelium versus adherent leukocytes. Cell adhesion data are expressed as number of cells per millimeter squared of venular surface, calculated from venular diameter and length, assuming cylindrical geometry.

\textbf{Blood–Brain Barrier Dysfunction}

Blood–brain barrier (BBB) permeability was assessed using the Evans blue (EB) extravasation method.\textsuperscript{16} A 2% solution of EB (Sigma-Aldrich) was injected (4 mL/kg) into the femoral vein immediately after reperfusion. Twenty-four hours later, 0.4 mL of blood was obtained by cardiac puncture and the mouse was transcardially perfused with phosphate-buffered saline (100 mm Hg) for 5 minutes. The brain was removed and separated from the dura mater and cerebellum. The cerebrum was divided into 2 hemispheres, each of which was homogenized and sonicated in 1 mL of 50% trichloroacetic acid (Sigma-Aldrich) and centrifuged at 10,000 rpm for 20 minutes. The supernatant was diluted with ethanol and the concentrations of EB in brain tissue and plasma were measured using a fluorescence spectrophotometer (FLUOstar Optima microplate reader; BMG LABTECH, Inc). BBB permeability was determined by dividing tissue EB concentration (\mu g/g brain weight) by the plasma concentration (\mu g/g).

\textbf{Brain Water Content}

At 24 hours after reperfusion in separate groups of mice, the brain was removed, stripped of the dura mater and cerebellum, and divided into 2 hemispheres. Each hemisphere was placed into a 60°C oven for 3 days to achieve complete desiccation. Water content was determined from (wet weight–dry weight)/wet weight and expressed as percent.

\textbf{Infarction Volume}

Twenty-four hours after reperfusion, infarction volume in 1-mm sections was evaluated using a 2,3,5-triphenyltetrazolium chloride method followed by correction of edema as previously described.\textsuperscript{17} All infarcts extended into both cortical and subcortical areas. The success rates of infarction in the mice that survived the procedure were: \textit{WT}=85.4\%, \textit{ob/ob}=82.1\%, \textit{ob/+}+\textit{Lep}=84.4\%.

\textbf{Cytokines in Plasma and Brain Tissue}

A cytometric bead array (Mouse Inflammation Kit; BD Biosciences) was used to measure the concentration of 6 cytokines (interleukin-12, tumor necrosis factor-\alpha, interferon-\gamma, monocyte chemoattractant protein-1 [MCP-1], interleukin-10, interleukin-6 [IL-6]) in postischemic plasma and brain tissue at 24 hours after reperfusion. Cytokine concentrations were expressed as either pg/mL (plasma) or pg/g brain weight (brain).

\textbf{Interleukin-6 and Monocyte Chemoattractant Protein-1 Immunoneutralization}

In some experiments, a blocking dose (2 mg/kg) of anti-mouse IL-6 or MCP-1 monoclonal antibody (R&D systems, Inc) was administered intraperitoneally 3 hours before the induction of ischemia.
Blood Glucose and Plasma Cholesterol Concentrations

After a 1-hour fast, blood was obtained from the tail and blood glucose concentration was measured using a handheld glucose reader (SureStep Meter; Lifescan Inc). Plasma cholesterol concentration was determined using a quantitative-enzymatic-colorimetric assay (Cholesterol LiquiColor Test; Stanbio Laboratory) and microplate scanning spectrophotometer.

Statistical Analysis

All data were expressed as mean±SE. Statistical difference between the different groups was determined by a 2-way analysis of variance with the Fisher post hoc test. A paired t test was used to compare responses between the right and left hemispheres. All analyses were performed using Statview software 4.5 (Abacus Concepts Inc). Statistical significance was set at \( P<0.05 \).

Results

Physiological Parameters

The Table summarizes the resting values of body weight, mean arterial blood pressure, plasma glucose and cholesterol concentrations, blood pH, \( O_2 \) saturation, and leukocyte and platelet counts in blood of WT, \( ob/ob \), and leptin-reconstituted \( ob/ob \) mice (\( ob/ob+Lep \)). No significant differences from WT mice were noted except for body weight and plasma cholesterol, which were higher in both \( ob/ob \) and \( ob/ob+Lep \) mice, but remained below 2.5 mmol/L (approximately 97 mg/dL). In \( ob/ob \) mice with an implanted Alzet pump, a plasma leptin concentration of 3.86±0.10 ng/mL was achieved, which compares to 3.25±0.67 ng/mL plasma concentration detected in WT mice at baseline (similar to previously reported WT values18). The 3-day leptin infusion resulted in a 73.3% reduction in food intake and a 5.2% reduction in body weight.

Table. Resting Values for Different Physiological Variables in WT Mice, \( ob/ob \) Mice, and \( ob/ob \) Mice Implanted With a Leptin-Loaded Alzet Pump (\( ob/ob+Lep \))

<table>
<thead>
<tr>
<th>Variable</th>
<th>WT</th>
<th>( ob/ob )</th>
<th>( ob/ob+Lep )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight, g</td>
<td>21.7±0.30</td>
<td>43.8±0.38</td>
<td>41.6±0.52</td>
</tr>
<tr>
<td>Mean arterial blood pressure, mm Hg</td>
<td>78.96±2.89</td>
<td>76.34±2.49</td>
<td>76.27±2.59</td>
</tr>
<tr>
<td>Glucose, mmol/L</td>
<td>10.8±0.33</td>
<td>10.9±0.42</td>
<td>10.2±0.60</td>
</tr>
<tr>
<td>Cholesterol, mmol/L</td>
<td>1.59±0.069</td>
<td>2.38±0.099</td>
<td>2.47±0.101</td>
</tr>
<tr>
<td>pH</td>
<td>7.277±0.019</td>
<td>7.245±0.042</td>
<td>7.240±0.026</td>
</tr>
<tr>
<td>( O_2 ) saturation, %</td>
<td>99.0±0.12</td>
<td>98.9±0.15</td>
<td>99.2±0.15</td>
</tr>
<tr>
<td>White blood cell count per microliter</td>
<td>3704±3.71</td>
<td>3013±2.29</td>
<td>3613±2.47</td>
</tr>
<tr>
<td>Platelet count ×10⁶/µL</td>
<td>82.8±5.9</td>
<td>79.4±1.1</td>
<td>84.3±10.2</td>
</tr>
</tbody>
</table>

Data are expressed as mean±SE.

Blood Glucose and Plasma Cholesterol Concentrations

After a 1-hour fast, blood was obtained from the tail and blood glucose concentration was measured using a handheld glucose reader (SureStep Meter; Lifescan Inc). Plasma cholesterol concentration was determined using a quantitative-enzymatic-colorimetric assay (Cholesterol LiquiColor Test; Stanbio Laboratory) and microplate scanning spectrophotometer.
Blood Cell–Vessel Wall Interactions
Figure 1 summarizes the blood cell–vessel wall interactions induced in cerebral venules by 30 minutes MCAO and 4-hour reperfusion in WT, ob/ob, and ob/ob/H11001 Lep mice. No significant differences in blood cell adhesion were noted between any of the sham-operated groups. In WT mice, MCAO/reperfusion significantly increased the number of adherent leukocytes (Figure 1A) and platelets (Figure 1B). The blood cell recruitment was further increased in ob/ob mice and ob/ob/H11001 Lep mice, but did not differ between ob/ob and ob/ob/H11001 Lep groups. The percentage of adherent leukocytes that were associated with platelets was 75% to 79%, whereas the fraction of adherent platelets that were attached to adherent leukocytes increased from 51% in WT mice to 66% in ob/ob mice.

Blood–Brain Barrier Dysfunction and Brain Edema
Figure 2 presents the changes in BBB permeability to EB (Figure 2A) and brain water content (Figure 2B) induced by MCAO/reperfusion in WT, ob/ob, and ob/ob/H11001 Lep mice at 24-hour reperfusion. No significant differences in EB leakage were noted between the left and right hemispheres of sham-operated WT, ob/ob, and ob/ob/H11001 Lep mice. However, MCAO/reperfusion produced a significant increase in EB leakage in the left hemisphere of WT mice without altered EB leakage in the left hemisphere of ob/ob/H11001 Lep mice.
accumulation in the right hemisphere. A much larger increase in EB leakage was noted in the left brain of ob/ob mice. A further increment in EB leakage was detected in leptin-reconstituted ob/ob mice. A similar pattern of changes in the left and right hemispheres was noted for tissue water content after MCAO/reperfusion between the different experimental groups.

Infarct Volume
An infarct volume of 16.39 ± 2.44% was detected in WT mice exposed to MCAO and 24-hour reperfusion (Figure 3). The infarct volume was significantly larger (28.65 ± 1.54%) in ob/ob mice, and an even larger increase was noted in leptin-reconstituted ob/ob mice.

Cytokine Levels
Of the cytokines tested, only MCP-1 and IL-6 exhibited significant changes in brain tissue and plasma after MCAO and 24-hour reperfusion (Figure 4). MCP-1 levels in the left hemisphere increased to a comparable extent in both WT and ob/ob mice (Figure 4A). Although IL-6 was increased above sham levels in the left hemisphere of both WT and ob/ob mice, a much smaller increment was detected in the obese group. Plasma levels of both MCP-1 and IL-6 were significantly increased after MCAO/reperfusion only in ob/ob mice (Figure 4B).

Immunoneutralization of Monocyte Chemoattractant Protein-1 and Interleukin-6
Because the plasma levels of both MCP-1 and IL-6 were significantly elevated after MCAO/reperfusion in ob/ob mice, we also determined whether blocking antibodies directed against either cytokine would attenuate infarct volume in ob/ob mice at 24-hour reperfusion. Figure 5 illustrates that...
immunoneutralization of MCP-1, but not IL-6, significantly reduced the MCAO/reperfusion-induced infarct volume in ob/ob mice.

Discussion
Epidemiological studies have revealed an increased risk for ischemic stroke in overweight or obese subjects that is independent of diabetes, hypertension, or hypercholesterolemia. This increased risk for stroke is accompanied by worse long-term prognosis and higher mortality. Despite the growing prevalence of obesity worldwide and its impact on the incidence and severity of ischemic stroke, little effort has been made to study ischemic stroke in animal models of obesity and to assess potential mechanisms underlying the deleterious effects of obesity. The present study provides evidence that leptin-deficient ob/ob mice exhibit more brain injury and inflammation in response to cerebral I/R than their lean counterparts. The exaggerated injury and inflammatory responses in obese mice appear to be independent of hypertension, hyperglycemia, and clinically significant hypercholesterolemia, which is consistent with obesity as an independent risk factor for cardiovascular disease.

Previous studies of focal and global brain ischemia indicate that the resultant brain injury is accompanied by an intense inflammatory response that includes the recruitment of adherent leukocytes and platelets in cerebral venules. This well-documented response, coupled with reports describing protection against ischemic stroke by interventions that prevent the adhesion of inflammatory cells, suggests that inflammation is a major component of the pathogenesis of ischemic stroke. The results of the present study indicate that the recruitment of adherent leukocytes and platelets in cerebral venules is more pronounced in ob/ob mice than in their lean counterparts. Such an exaggerated inflammatory and prothrombogenic phenotype in cerebral microvessels of obese (ob/ob) mice has been previously demonstrated in a cecal ligation and puncture model of sepsis, in which the recruitment of adherent platelets and leukocytes in cerebral venules, increased P-selectin expression, and behavioral deficits elicited by sepsis were more pronounced in ob/ob mice. After ischemic stroke in ob/ob mice, the increased platelet accumulation in cerebral venules primarily reflects the binding of platelets to adherent leukocytes rather than enhanced adhesive interactions between platelets and venular endothelium. This response may result from increased platelet activation and an associated increase in platelet P-selectin, which would promote the adhesion of platelets to leukocyte P-selectin glycoprotein ligand 1. Whether the more pronounced platelet adhesion response observed in obese mice leads to an elevated risk of occlusive events that propagate the injury response remains unclear. However, the growing body of evidence that intimately links inflammation and thrombosis is consistent with such a scenario in the postischemic brain.

In addition to the increased avidity of cerebral venules for adherent leukocytes and platelets, the cerebral microvascular dysfunction elicited by I/R can be manifested as impaired BBB function. An increased permeability of cerebral microvessels has the potential to promote brain edema. Although lean (WT) mice exposed to 30 minutes of MCAO and 24 hours reperfusion exhibited a 3-fold increase in EB leakage, a significantly larger increment (6-fold) in vascular leakage was noted after MCAO/reperfusion in ob/ob mice. The enhanced MCAO/reperfusion-induced BBB dysfunction in ob/ob mice was associated with a corresponding increase in brain water content. BBB dysfunction has been previously described in obese Zucker rats and in patients with metabolic syndrome; however, this study provides the first evidence for obesity-related alterations in BBB function in the absence of hyperglycemia. Although the larger infarction volume detected in ob/ob mice in the present study has not been previously reported for a model of ischemic stroke, studies of myocardial I/R in insulin resistant Zucker obese rats have demonstrated significantly larger myocardial infarcts compared with those detected in Zucker lean rats. However, the larger infarct volume that we noted in the brain of ob/ob cannot be attributed to hyperglycemia and the development of type II diabetes. It is interesting to note that the exacerbated cerebral infarct volume in obese mice was apparent as early as 24 hours after reperfusion. Whether the difference in infarct volumes between lean and obese mice would increase, decrease, or remain the same at longer times of reperfusion remains unclear. Resolution of this issue may be difficult if ob/ob mice are less likely to tolerate longer reperfusion periods.

The ob/ob mouse strain lacks the gene for leptin, which regulates food intake. This appetite-repressing peptide is produced by white adipose tissue and its plasma concentration is normally proportional to body fat. Leptin also exerts an influence on other physiological processes, including the innate and adaptive immune systems. If and how leptin influences inflammation remains controversial with some studies suggesting that leptin is proinflammatory, whereas others invoke an antiinflammatory action. Leptin appears to promote the activation of T-lymphocytes, which have been implicated in the microvascular dysfunction and tissue injury induced by MCAO/reperfusion in mice. To determine whether the exaggerated inflammatory and injury responses to MCAO/reperfusion noted in ob/ob mice is due to leptin deficiency, we acutely restored plasma leptin in ob/ob mice to normal levels using Alzet pumps without significantly altering adipose tissue volume. These studies revealed that leptin deficiency per se does not account for the exaggerated inflammatory and injury responses because leptin reconstitution did not afford protection, but instead resulted in further exaggeration of the responses. Our findings are therefore consistent with the view that leptin is a proinflammatory molecule in the setting of obesity and suggest that normal or elevated levels of leptin, when combined with obesity, may lead to an amplification of the injury response to I/R. In lean mice, acute administration of leptin has been shown to confer protection against ischemia-induced cerebral infarcts. An explanation for the qualitatively different responses of lean and obese mice to leptin administration is not readily available, but it may reflect an influence of adipose tissue mass on the injury response.

Of the panel of cytokines/chemokines measured in plasma after MCAO/reperfusion, only IL-6 and MCP-1 were more profoundly elevated in ob/ob mice compared with lean mice.
Because brain tissue levels of these cytokines were either similar (MCP-1) or lower (IL-6) in postischemic ob/ob mice compared with WT mice exposed to MCAO/reperfusion, it is unlikely that the higher plasma levels were derived from the damaged brain. However, it is possible that the higher plasma concentrations in ob/ob mice were derived from the expanded pool of adipose tissue, because both MCP-1 and IL-6 are produced and secreted by adipose tissue.\textsuperscript{5,33} Immunoneutralization of MCP-1, but not IL-6, significantly reduced the brain injury (infarct volume) elicited by MCAO/reperfusion in ob/ob mice, suggesting that the elevated plasma level of MCP-1 contributes to the injury process. Plasma MCP-1 concentration has been reported to be 2-fold higher in patients with stroke than in control subjects.\textsuperscript{11} Furthermore, it has been previously reported that lean mice receiving either a MCP-1-blocking antibody\textsuperscript{34} or gene transfer of dominant negative MCP-1\textsuperscript{35} as well as MCP-1 knockout mice\textsuperscript{36} are protected against ischemic brain injury. Similarly, MCP-1-overexpressing transgenic mice exhibit an exacerbation of ischemic brain injury.\textsuperscript{37} These reports, coupled to our findings in obese mice indicate that MCP-1 blockade may be a useful therapeutic strategy in both lean and obese patients with stroke. Conversely, it is not surprising that blockade of IL-6 did not reduce the infarct volume in ob/ob mice because it has been suggested that this cytokine is neuroprotective in the postischemic brain.\textsuperscript{38} Hence, the blunted increase (compared with WT mice) in brain IL-6 levels observed in postischemic ob/ob mice may also contribute to the exacerbated injury response in these mice.

In conclusion, the present study provides evidence that obese mice exhibit more pronounced inflammatory and brain injury responses to ischemia and reperfusion than their lean counterparts. The exaggerated inflammatory/injury responses in ob/ob mice do not appear to result from either leptin deficiency or the elevated plasma IL-6 levels that are elicited by MCAO/reperfusion. However, exaggerated increases in the circulating levels of MCP-1 do appear to have important pathological consequences in determining the larger infarct size associated with obesity. Our findings underscore the need for additional work that addresses the influence of obesity on the severity of ischemic stroke and the mechanisms that underlie the deleterious effects of this cardiovascular risk factor.

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Disclosures
None.

References


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