Stroke

BASIC AND TRANSLATIONAL SCIENCES

Aflibercept, a VEGF (Vascular Endothelial Growth Factor)-Trap, Reduces Vascular Permeability and Stroke-Induced Brain Swelling in Obese Mice

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BACKGROUND AND PURPOSE: Brain edema is an important underlying pathology in acute stroke, especially when comorbidities are present. VEGF (Vascular endothelial growth factor) signaling is implicated in edema. This study investigated whether obesity impacts VEGF signaling and brain edema, as well as whether VEGF inhibition alters stroke outcome in obese subjects.

METHODS: High-fat diet-induced obese mice were subjected to a transient middle cerebral artery occlusion. VEGF-A and VEGFR2 (receptor) expression, infarct volume, and swelling were measured 3 days post-middle cerebral artery occlusion. To validate the effect of an anti-VEGF strategy, we used aflibercept, a fusion protein that has a VEGF-binding domain and acts as a decoy receptor, in human umbilical vein endothelial cells stimulated with rVEGF (recombinant VEGF; 50 ng/mL) for permeability and tube formation. In vivo, aflibercept (10 mg/kg) or IgG control was administered in obese mice 3 hours after transient 30 minutes middle cerebral artery occlusion. Blood-brain barrier integrity was assessed by IgG staining and dextran extravasation in the postischemic brain. A separate cohort of nonobese (lean) mice was subjected to 40 minutes middle cerebral artery occlusion to test the effect of aflibercept on malignant infarction.

RESULTS: Compared with lean mice, obese mice had increased mortality, infarct volume, swelling, and blood-brain barrier disruption. These outcomes were also associated with increased VEGF-A and VEGFR2 expression. Aflibercept reduced VEGF-A-stimulated permeability and tube formation in human umbilical vein endothelial cells. Compared with the IgG-treated controls, mice treated with aflibercept had reduced mortality rates (40% versus 17%), hemorrhagic transformation (43% versus 27%), and brain swelling (28% versus 18%), although the infarct size was similar. In nonobese mice with large stroke, aflibercept neither improved nor exacerbated stroke outcomes.

CONCLUSIONS: The study demonstrates that aflibercept selectively attenuates stroke-induced brain edema and vascular permeability in obese mice. These findings suggest the repurposing of aflibercept to reduce obesity-enhanced brain edema in acute stroke.

GRAPHIC ABSTRACT: An online graphic abstract is available for this article.

Key Words: brain edema ■ comorbidity ■ obesity ■ stroke ■ vascular endothelial growth factor

Stroke is a major cause of death and the leading cause of physical disability worldwide. Despite numerous neuroprotective targets identified in animal models of stroke, neuroprotection strategies have had little or no efficacy in controlled clinical trials.[1–3] Factors that underlie this lack of translational success include a lack of cardiovascular and cerebrovascular risk factors in animal models that are common in human patients.[4,5] In addition, the heavy reliance on infarct reduction as a gauge of neuroprotection in preclinical studies without

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Nonstandard Abbreviations and Acronyms

Ang2	angiopoietin-2
AQP4	aquaporin 4
BBB	blood-brain barrier
FITC	fluorescein isothiocyanate
HD	high-fat diet
MCAO	middle cerebral artery occlusion
NRP-1	neuropilin-1
PIGF	placental growth factor
rVEGF-A	recombinant vascular endothelial growth factor
SUR1	sulfonylurea receptor 1
Tie2	Tek tyrosine kinase
TRPM4	transient receptor potential melastatin 4
VEGFR2	vascular endothelial growth factor receptor 2

considering brain edema has likely hindered translational success. Brain edema is an acute pathological event in stroke that leads to blood-brain barrier (BBB) disruption, inflammation, ionic dysregulation, hemorrhagic transformation, and increased mortality.[6,7] Moreover, stroke-induced edema also elevates blood glucose levels, hemispheric volume, and local compartmental pressure in patients.[8,9] The advent of edema in acute stroke is a fatal complication during infarct development, and it is associated with high mortality and morbidity.[8,10,11]

Brain swelling from stroke-induced edema derives from the disruption of cellular ionic balance and inflammation. Cytotoxicity resulting from edema is typically followed by vasogenic edema that increases vascular permeability and new vessel formation.[12,13] Enhanced vascular permeability associated with edema involves the activation of VEGF (vascular endothelial growth factor).[14] The VEGF family contains several members (eg, VEGF-A, -B, -C, and PIGF [placental growth factor]), but it is the interaction of VEGF-A with VEGFR2 (VEGF receptor 2) that has been implicated in regulating angiogenesis, neuroprotection, neurogenesis, and vascular permeability during homeostasis and pathological conditions.[15–18]

Following the stroke, VEGF-A expression increases in astrocytes, endothelial cells, and neurons, whereas endothelial cells in the peri-infarct area express VEGFR2.[15,19] Administration of exogenous rVEGF165 (a VEGF-A isoform) in animal stroke models provides neuroprotection, enhances angiogenesis, and improves neurological deficits in stroke,[20–22] which is consistent with postmortem analyses that suggested promoting angiogenesis in the penumbra could improve stroke patient outcomes.[23] When comorbid conditions are present, however, excessive angiogenesis in the poststroke brain is correlated with adverse outcomes in patients.[24] In diabetic animals, for example, increased brain swelling was linked to VEGF signaling, and blocking this signaling reduced brain edema.[25] Given that VEGF signaling in the ischemic brain is linked with brain edema as well as increased vascular permeability and intracranial pressure,[14,26] then the development of anti-VEGF strategies may be effective in counteracting comorbidity-enhanced vascular permeability and brain edema in acute stroke.

In this study, we investigated the impact of obesity on acute stroke outcomes and whether an anti-VEGF agent could reduce obesity-enhanced brain edema and improve acute stroke outcomes. Obesity was used as a comorbid condition because it is one of the most common comorbidities in stroke, and it leads to a host of metabolic disturbances (such as insulin resistance, diabetes, dyslipidemia, and elevated blood pressure) that elevate stroke risk. Additionally, obesity induces inflammation, vascular dysfunction, and exacerbate injury responses after brain ischemia.[27–30] To model obesity, diet-induced obesity was used because it closely mimics human obesity. To target VEGF signaling, we used aflibercept (VEGF-trap), which is a recombinant fusion protein with a VEGF-binding domain that acts as a decoy receptor for all diffusible isoforms of VEGF-A, VEGF-B, and PIGF.[31] As a Food and Drug Administration-approved therapeutic agent, aflibercept was developed to treat age-related macular degeneration, diabetic macular edema, retinopathy of prematurity, and cancers.[32–35] Our study shows that aflibercept reduces poststroke mortality, brain swelling, and BBB disruption without affecting infarct size in obese stroke mice. In addition, we show that aflibercept does not adversely affect malignant infarction in lean mice. Together, these findings suggest aflibercept is safe for treatment to reduce obesity-enhanced brain edema in acute stroke.

METHODS

The data that support the findings of this study are available from the corresponding author upon reasonable request. Detailed descriptions of the Materials and Methods are provided in the Data Supplement.

Animals

Procedures for the use of animals were approved by the Institutional Animal Care and Use Committee of Weill Cornell Medicine in accordance with the Institutional Animal Care and Use Committee, National Institutes of Health, and Animal Research: Reporting of In Vivo Experiments guidelines.

Diet-Induced Obesity Mouse Model

C57BL/6 mice (8-week-old) were fed a normal diet (LabDiet, St Louis, MO) to generate nonobese (lean) mice or a high-fat diet (HD, Bioserv, Flemington, NJ) for either 8 weeks (males) or 12 weeks (females) to obtain similar obesity phenotypes. One week before the end of diet intervention, glucose tolerance tests were performed in overnight fasted mice.

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Transient Middle Cerebral Artery Occlusion

Mice were subjected to 30 or 40 minutes of transient middle cerebral artery occlusion (MCAO), and poststroke care was performed as described previously.[36]

Assessment of Permeability

For the in vitro assays, permeability was assessed with human umbilical vein endothelial cells (Lonza, Walkersville, MD) that were stimulated with human rVEGF (Sigma-Aldrich). Permeability was performed by measuring the amount of fluorescein isothiocyanate (FITC)-dextran (Sigma-Aldrich). For the in vivo assays, these were performed as previously described.[25] Briefly, frozen brain sections (20 μm thickness) were incubated with anti-mouse IgG antibody (1:1000; Vector Laboratories; BA-9200) to visualize IgG staining. To determine the extravasation of dextran, FITC-labeled dextran was injected retro-orbitally 3 hours before euthanize, and dextran staining intensity was assessed. Quantification was done using the Image J software.

Assessment of VEGF-A and VEGFR2 mRNA and VEGFR2+ Cells

Samples were prepared from the contralateral and ipsilateral hemispheres, excluding the olfactory bulb and cerebellum. Gene expression was quantified with real-time reverse transcription-polymerase chain reaction using fluorescent TaqMan technology, as has been previously described.[25] The number of $VEGFR2^+$ cells and the intensity of VEGFR2 expression in the brain endothelial cells were determined by flow cytometry.

Statistics

All in vivo data are expressed as the mean±95% CI, whereas all in vitro data are expressed as the mean±SEM. The sample size for in vivo studies (minimum n=10/group) was determined based on predicting detectable differences to reach the power of 0.80 at a significance level of <0.05, assuming a 33% difference in mean and a 25% SD at the 95% CI. For in vivo studies, the Student *t* test was used to compare differences between 2 groups (ie, infarct size, swelling, and permeability assay). To determine the survival curve, a log-rank test was performed. Multigroup analyses were performed using either 1-way (in vitro studies) or 2-way (gene expression studies) ANOVA using Prism 9.0. Bonferroni post hoc comparisons of the ANOVA were used to compare either genotype and stroke effects or genotype and treatment effects. Normality of data was analyzed using the D'Agostino-Pearson test. The level of significance was set at $P<0.05$.

RESULTS

Diet-Induced Obese Mice Display Delayed Glucose Clearance

To model human obesity, mice were fed an HD. Male mice were fed an HD for 8 weeks, whereas female mice were fed an HD for 12 weeks to obtain an obese phenotype similar to the males. The HD feeding significantly increased body weight gain in both males and females when compared with mice fed a normal diet (Figure IA in the Data Supplement). HD-induced obese mice of both sexes developed insulin resistance as indicated by their significantly slower glucose clearance (Figure IB in the Data Supplement).

Obesity Exacerbates Acute Stroke Outcomes and Enhances BBB Disruption

Following ischemic stroke, obese mice displayed significantly increased mortality when compared with lean mice (17% versus 45%; Figure 1A). Obese mice also had a higher incidence of hemorrhagic transformation (18% versus 44%; Figure 1B). The hemorrhagic transformation included parenchymal hematoma and hemorrhagic infarction in either the penumbra or infarct core.[37] Histological analyses revealed increased infarct volume and edema in obese mice (Figure 1C). In lean mice, infarct size significantly correlated with edema (r^2=0.3913, $P<0.05$) but not in obese mice (r^2=0.0073, ns; Figure 1C). The lack of correlation between infarct size and edema in obese mice suggests that obesity-enhanced brain edema is an injury-independent acute pathological event (Figure 1C). Moreover, obesity-enhanced edema was accompanied by greater vascular permeability, as revealed by increased IgG staining and dextran extravasation (Figure 1D). The extent of dextran extravasation (as an indication of BBB disruption) at 3 days postischemia was correlated with infarct volume only in lean (P=0.047), but not obese, mice. Importantly, the dextran+ area was significantly correlated with brain swelling only in obese mice (P=0.0006, Figure II in the Data Supplement). Together, these results show that obesity adversely impacts acute stroke outcomes and that brain swelling is the predominant pathological event with this comorbid condition.

Obesity Increases Stroke-Induced VEGF-A and VEGFR2 Expression in the Brain

VEGF signaling is a key pathway for modulating angiogenesis and vascular permeability.[14,15] To determine whether the VEGF pathway following stroke was altered by obesity, both VEGF-A and VEGFR2 expression levels in the brain were determined at 3 days poststroke. VEGF-A and VEGFR2 gene expression levels were the same in either the ipsilateral or contralateral hemisphere of lean mice following stroke (Figure 2A and 2B). By contrast, the expression levels for both genes were significantly and selectively increased in the ipsilateral hemisphere of obese mice (Figure 2A and 2B). Because endothelial cells express $CD31^+$ but do not express CD45, $VEGFR2^+$ cells were determined in $CD31^+/CD45^-$ endothelial cells. Consistent with VEGFR2 gene expression, flow cytometry analysis showed significantly more $VEGFR2^+$ cells in the ipsilateral hemisphere of obese mice when compared with lean mice (indicated by a circle, Figure 2D).

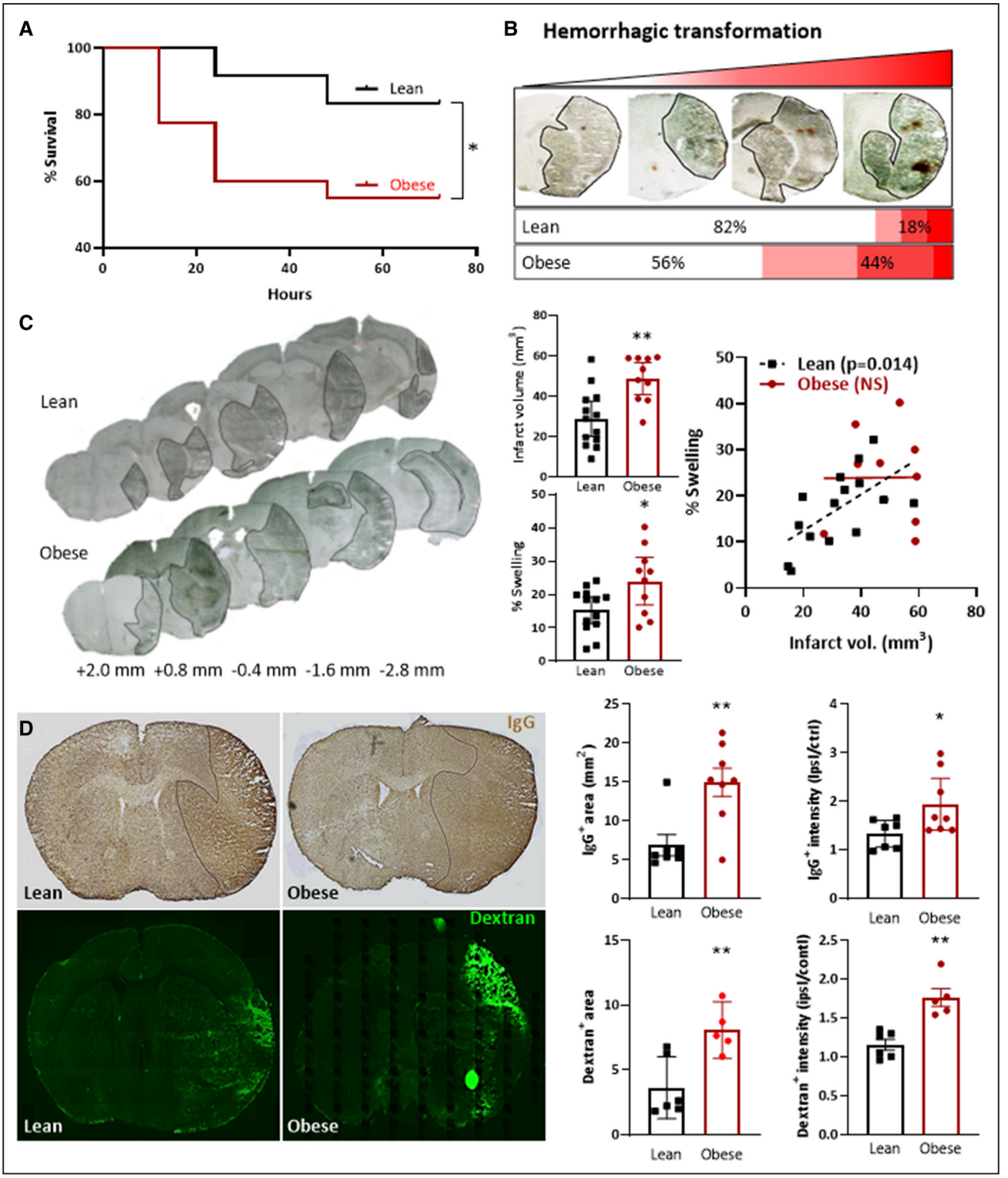

Figure 1. Impact of obesity on stroke outcomes.
A, Survival curve for 72 h following transient middle cerebral artery occlusion (MCAO) shows increased mortality in obese mice (18 died out of 40) compared with lean mice (4 died out of 24); asterisk indicates $P<0.05$. **B**, Obese mice had increased rates of hemorrhagic transformation (which included hemorrhagic infarction [light red] and parenchymal hematoma [dark red]; n=10 and 13 for lean and obese, respectively). Arrowheads indicate petechial bleeding, and the arrow identifies hematoma. **C**, Obese mice had larger average infarct volumes and swelling when compared with lean mice. These outcomes were not correlated in individual obese mice while they were in individual lean mice (n=10 and 13 for lean and obese mice, respectively). **D**, IgG immunostaining (**upper**) and fluorescein isothiocyanate (FITC)-labeled dextran extravasation (**lower**) reveal increased vascular permeability in the obese mice (n=6 and 8 for lean and obese, respectively). *, **$P<0.05$, 0.01 vs lean.

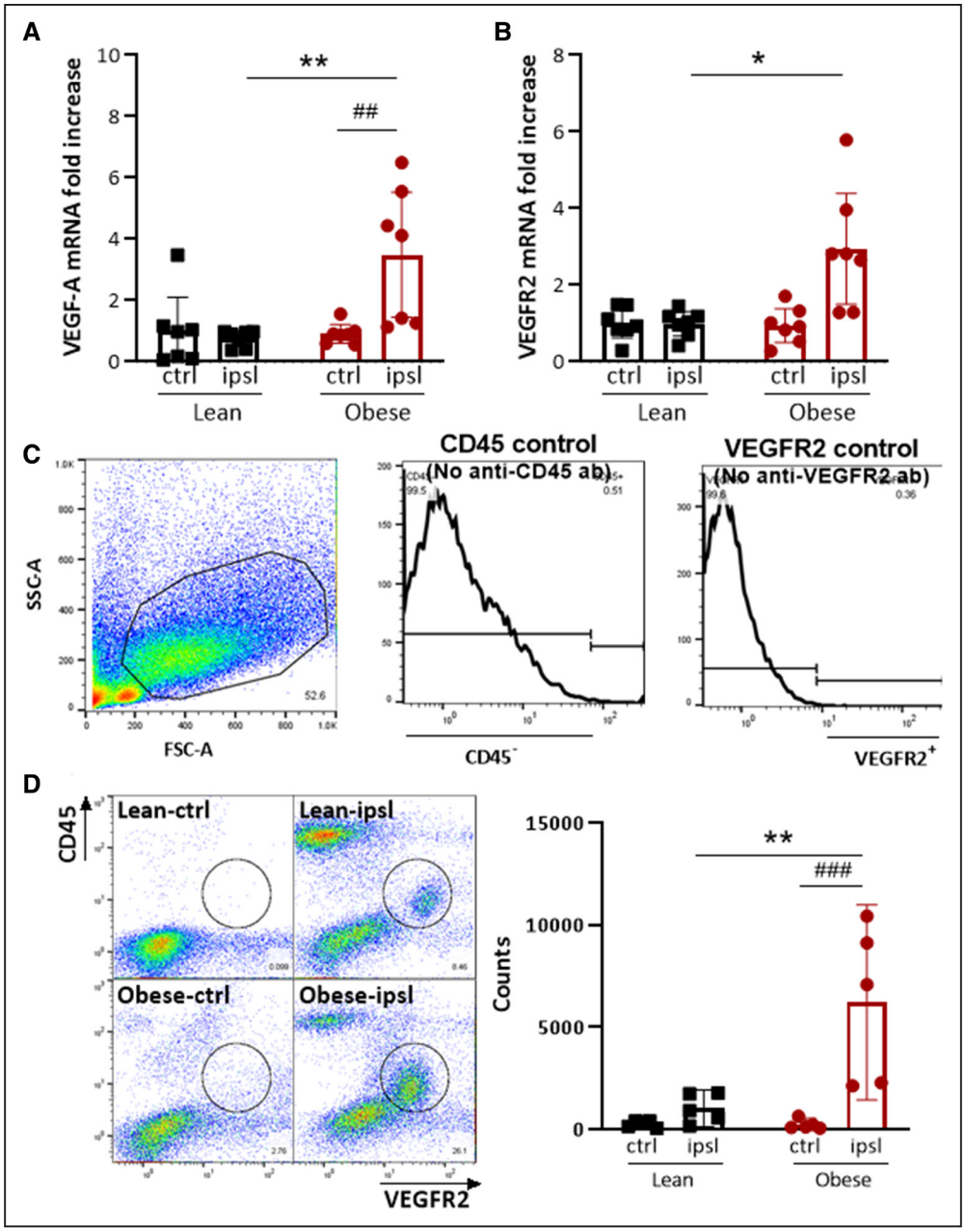

Figure 2. Effect of obesity on stroke-induced VEGF (vascular endothelial growth factor)-A and VEGFR2 (VEGF-A and receptor) expression in the brain.

A and **B**, VEGF-A and VEGFR2 mRNA levels at 3 d post-middle cerebral artery occlusion (MCAO; n=7/group), respectively. **C** and **D**, Flow cytometry analysis of VEGFR2$^+$ endothelial cells in the postischemic brain. **C**, Endothelial cells express CD31$^+$ but do not express CD45. Scattered plot of isolated CD31$^+$ cells from each hemisphere by CD31$^+$ magnetic beads. Antibody controls to identify CD45 and VEGFR2 positive staining. **D**, Circles indicate CD45$^-$ and VEGFR2$^+$ cells in CD31$^+$ population. Flow cytometry and quantification of VEGFR2$^+$ cells in CD31$^+$/CD45$^-$ endothelial cell population. n=5/group. ctrl indicates contralateral; and ipsl, ipsilateral. *, **P<0.05, 0.01 vs lean, ##, ###P<0.01, 0.001 vs ctrl.

Aflibercept Reduces VEGF-A-Induced Permeability and Tube Formation in Human Umbilical Vein Endothelial Cells

To assess whether excessive activation of VEGF signaling underlies the exacerbated stroke outcomes in obese mice, we tested the effects of aflibercept, which is a Food and Drug Administration-approved anti-VEGF drug that binds with high affinity to all VEGF isoforms.[35] We first determined the effects of aflibercept on permeability and angiogenesis in human umbilical vein endothelial cell cultures. Compared with control cultures, rVEGF treatment significantly increased permeability of dextran (70 kDa) in a dose-dependent manner after either 3 or 6 hours of incubation (Figure 3A). In cultures treated with aflibercept, however, rVEGF-induced dextran permeability was significantly reduced (Figure 3B). Tube formation in human umbilical vein endothelial cell cultures was also

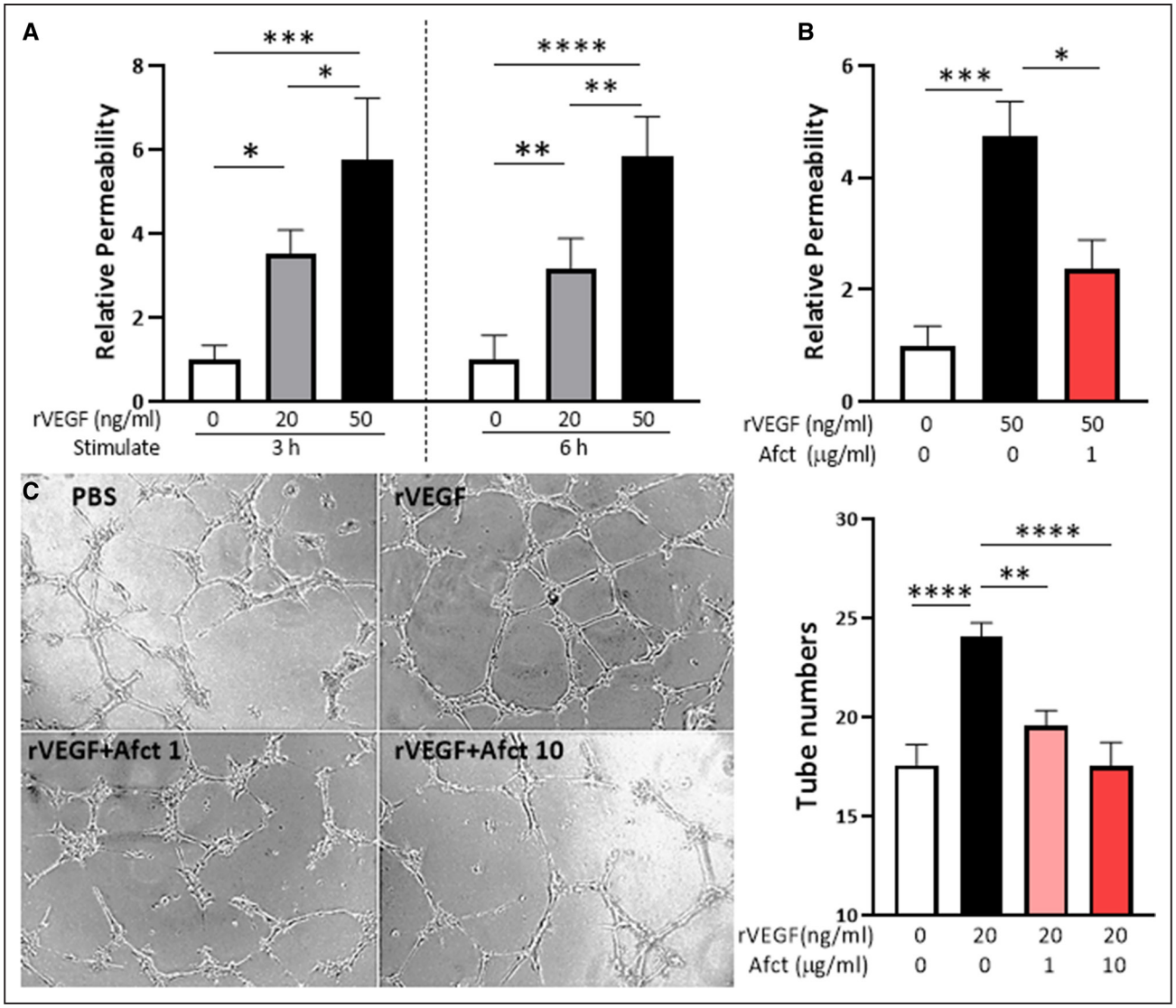

Figure 3. Effect of aflibercept on rVEGF (recombinant vascular endothelial growth factor)-induced permeability and tube formation in human umbilical vein endothelial cells (HUVECs).
A, HUVECs were cultured confluent monolayer in inserts and stimulated with rVEGF for either 3 or 6 h before permeability of the monolayer was determined with fluorescein isothiocyanate (FITC)-labeled dextran (70 kDa). **B**, HUVECs were stimulated with rVEGF for 3 h either in the presence or absence of aflibercept (1 μg/mL). **C**, Images and quantification of tube formation. Quantification of tube formation HUVECs stimulated with rVEGF for 10 h in the presence of either low or high doses of aflibercept (1 or 10 μg/mL) showed that aflibercept dose-dependently impeded tube formation (n=3 for each condition). *, **, ***, **** indicates $P<0.01$, 0.05, 0.001, 0.0001 vs each control, respectively.

significantly reduced by the presence of 1 or 10 μg/mL aflibercept (18.8±3.3% and 27.1±5.0%, respectively; Figure 3C). Together, these cell culture findings confirmed that pharmacological inhibition of VEGF signaling by aflibercept can effectively reduce VEGF-regulated processes, such as permeability and angiogenesis.

Aflibercept Reduces Stroke-Induced VEGF-A and VEGFR2 Expression in Obese Mice

To determine if aflibercept could attenuate the stroke-induced increase in VEGF signaling in obese mice, we administered either IgG (control) or 10 mg/kg of aflibercept at 3 hours posttransient MCAO. Analysis of VEGF-A and VEGFR2 gene expression levels at 3 days poststroke showed a significant reduction in the ipsilateral hemispheres of aflibercept-treated mice (Figure 4A and 4B). In addition, flow cytometry analysis showed that aflibercept treatment also reduced the number of VEGFR2$^+$ endothelial cells in the ipsilateral hemisphere (Figure 4C).

To further explore that action of aflibercept, NRP-1 (neuropilin-1) expression was examined. NRP-1 is a protein that interacts with VEGFR2 to promote endothelial tip cell function during angiogenesis.[38] Flow cytometry analysis revealed that the number of NRP-1$^+$/CD31$^+$ endothelial cells was also significantly reduced in obese mice treated with aflibercept (Figure III in the Data Supplement). Collectively, these results demonstrate in vivo

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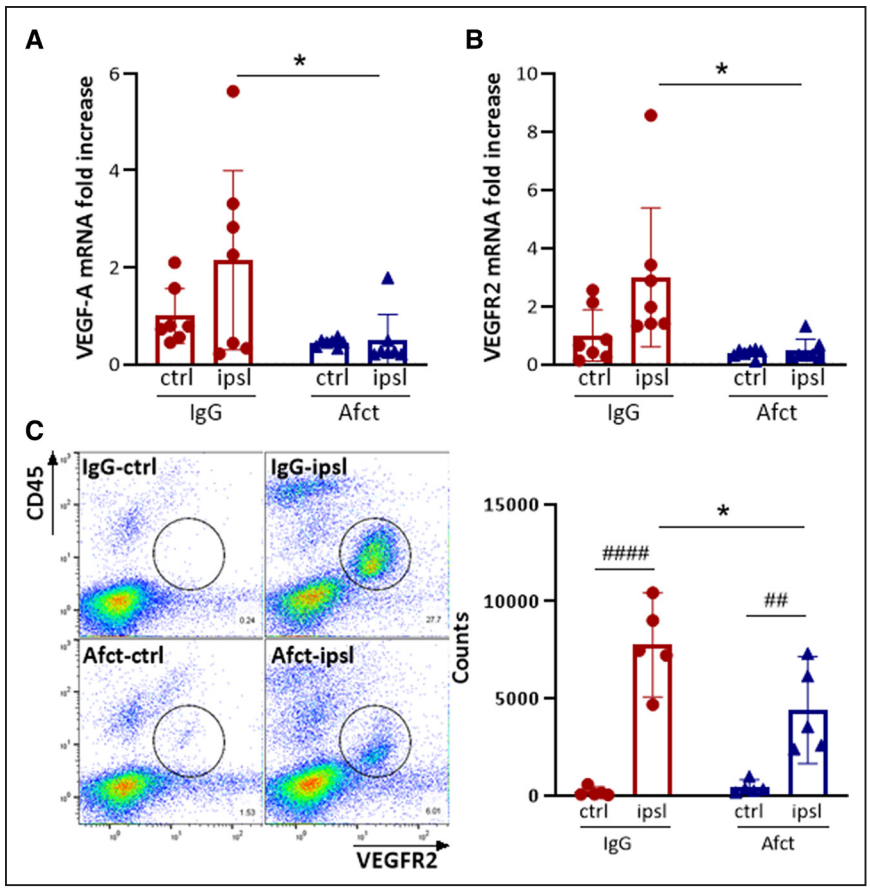

Figure 4. Effect of aflibercept on stroke-induced VEGF (vascular endothelial growth factor)-A and VEGFR2 (VEGF-A and receptor) expression in obese mice.
A and **B**, Brain VEGF-A and VEGFR2 mRNA levels, respectively, in obese mice 3 d after middle cerebral artery occlusion (MCAO; n=7/group). **C**, Flow cytometry of $CD31^+$ endothelial cells isolated from the poststroke brain using a CD31 magnetic beads. Circle indicates the number of $VEGFR2^+$ cells in $CD45^-/CD31^+$ endothelial cells. Note that the number of $VGFR2^+$ cells in the ipsilateral (ipsl) hemisphere was significantly reduced in aflibercept-treated obese mice (n=5/group). ctrl indicates contralateral. *P<0.05 vs IgG, ##, #### P<0.01, 0.0001 vs ctrl.

efficacy of aflibercept to reduce VEGF signaling activity in obese stroke.

Aflibercept Improves Poststroke Survival, Reduce Brain Edema, and BBB Disruption in Obese Mice

Aflibercept-treated (and IgG-treated control) obese mice subjected to transient MCAO were also examined to address whether inhibition of VEGF signaling could improve obesity-induced adverse stroke outcomes. Compared with IgG-treated mice, administration of aflibercept decreased both mortality (40% versus 17%) and the incidence of hemorrhagic transformation (43% versus 27%; Figure 5A). Moreover, aflibercept-treated mice had better neurological behavior assessments at 1 and 3 days after MCAO (Figure 5B). Histological analyses also revealed that aflibercept also significantly reduced brain edema without affecting infarct size (Figure 5C), which suggested that BBB permeability was reduced in aflibercept-treated mice. Consistent with this possibility, both IgG staining and extravasation of infused dextran were significantly reduced in the aflibercept-treated group (Figure 5D). Together, these findings indicate that the ability of aflibercept to improve stroke outcomes in obese mice is mediated, at least in part, by reducing brain edema and improving the preservation of BBB integrity.

Aflibercept Did Not Cause Adverse Outcomes in Malignant Infarct in Nonobese (Lean) Mice

Given that brain edema is a prominent feature in malignant infarct (Figure 1C), we addressed whether VEGF signaling contributes to exacerbating stroke outcomes following a severe stroke in lean mice. Since infarct size varies widely, we subcategorized mice that were subjected to 30 minutes MCAO and divided into 2 groups with moderate (20< and <50 mm^3) or large (>55 mm^3) infarct volumes. When compared with the moderate infarct size group, mice with large infarcts had significantly greater brain swelling and increased VEGFR2 mRNA levels in the stroked hemisphere (Figure IV in the Data Supplement). Together, these findings suggested that the level of stroke-induced VEGF signaling scales with the infarct size in lean mice.

To test the involvement of VEGF signaling on malignant infarction in nonobese mice, lean mice were subjected to MCAO for a longer duration (40 minutes). This occlusion time increased infarct size, brain edema, and VEGFR2 mRNA expression (Figure 6A and 6B). To address whether antagonizing VEGF signaling could reduce malignant stroke in lean mice, aflibercept or IgG was administered at 3 hours poststroke. In contrast to obese mice, our analysis at 3 days poststroke found aflibercept neither reduced infarct size nor brain swelling in lean mice with malignant stroke (Figure 6A). This absence of an

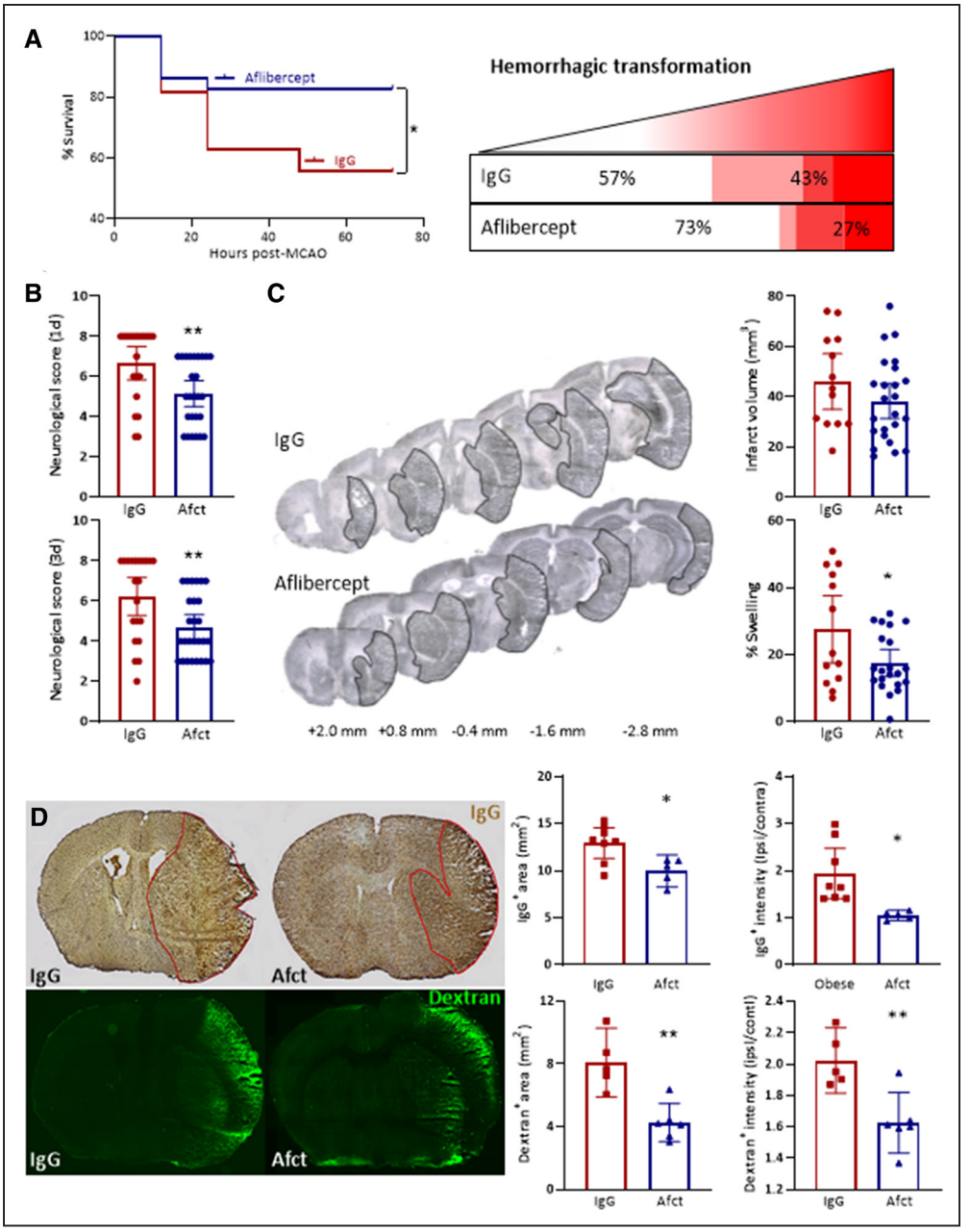

Figure 5. Effect of aflibercept on acute stroke outcomes in obese mice.
A, **Left**, Survival curve for 72 h following transient middle cerebral artery occlusion (MCAO) in obese mice shows reduced mortality in aflibercept-treated mice (5/29 died) compared with control IgG-treated mice (12/27 died). **Right**, Incidence of hemorrhagic transformation at 3 d poststroke in survived animals. **B**, Neurological impairment score determined at 1 and 3 d after MCAO. **C**, Histological analyses for infarct volume and hemispheric brain swelling assessed 3 d after MCAO. **D**, Assessment of vascular permeability by IgG immunostaining (**upper**) and fluorescein isothiocyanate (FITC)-labeled dextran extravasation (**lower**; n=5–6./group). *, **P<0.05, 0.01, vs IgG.

effect on either infarct volume or swelling was not due to a lack of drug activity since the aflibercept-treated mice did have reduced VEGFR2 gene expression levels (Figure 6B). Also, in contrast to obese mice, dextran assays showed that aflibercept did not improve BBB integrity in lean mice with malignant stroke (Figure 6C). Survival of aflibercept-treated mice showed a trend higher survival during the first 48 hours after stroke, but the overall survival rate for 3 days after stroke was not statistically different between control (IgG) and aflibercept-treated groups (Figure 6D). Together, these results show that the effects of aflibercept are neutral in lean mice, in that it did not adversely impact severe stroke, but in contrast to obese mice, it also did not improve severe stroke outcomes as measured by infarct size and swelling.

DISCUSSION

Comorbidities are an important consideration for determining the appropriate treatment of ischemic stroke.

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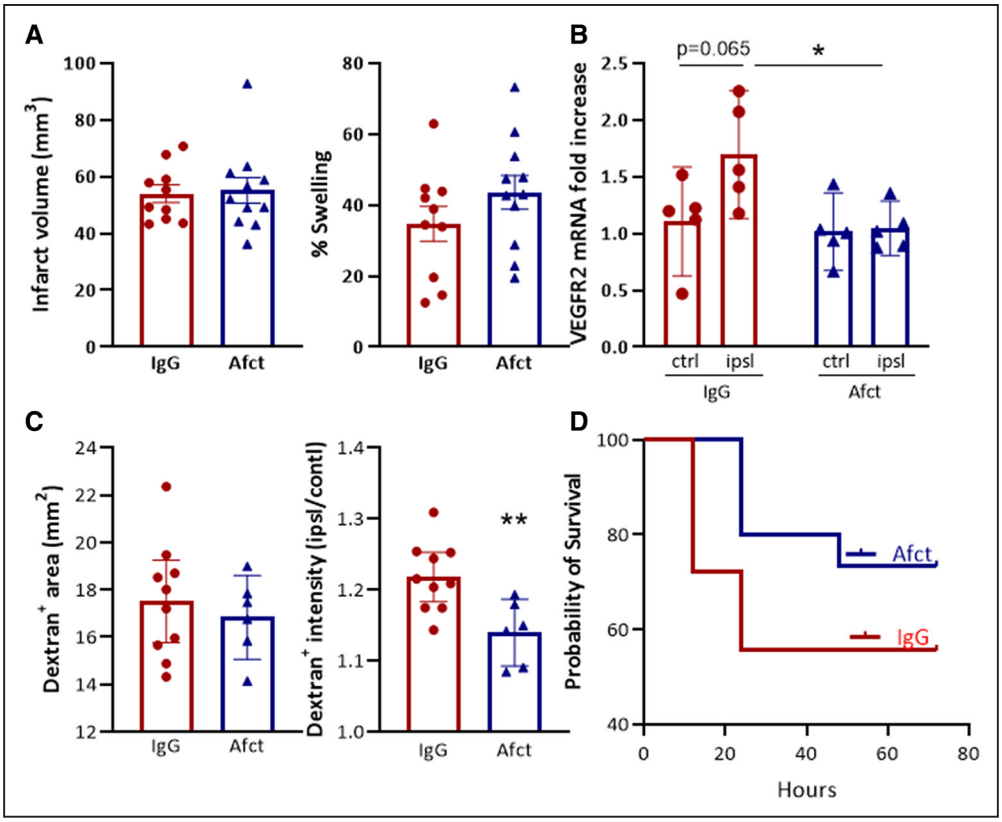

Figure 6. Effect of aflibercept on acute stroke outcomes in nonobese mice with severe stroke.
Mice subjected to 40 min middle cerebral artery occlusion (MCAO; severe stroke) were treated with IgG control or aflibercept and outcome analyses at 3 d poststroke. **A**, Assessment of infarct volume and percent hemispheric swelling. **B**, VEGFR2 (Vascular endothelial growth factor A and receptor) mRNA levels. **C**, Blood-brain barrier (BBB) permeability assessment. N=4–6/group. **D**, Survival curve. Eight out of 18 (IgG) and 4 out of 15 (aflibercept) were died for 3 d postischemia. *, ** $P<0.05$, 0.01 vs IgG.

Previously studies demonstrated that stroke with hyperlipidemic and diabetic comorbidities disproportionately enlarged brain swelling when compared with the corresponding infarct size.[25,39,40] This previous disparity in pathological outcomes between normal and comorbid metabolic conditions motivated the present investigation to address whether brain edema is a critical stroke pathology when comorbidities are present. Since obesity is a predisposing condition that underlies a host of metabolic dysfunction in humans, the current study used a mouse model of diet-induced obesity rather than a genetically altered mouse line to more closely recapitulate the features of human obesity. Using this mouse model of obesity, our study addressed the impact of obesity on acute stroke outcome and mechanisms underlying associated brain edema, as well as an intervention strategy.

Previous preclinical studies, in general, have not emphasized brain swelling in stroke outcome because stroke-induced brain swelling is transient and its inclusion results in an overestimate of infarct volume. Accordingly, to assess neuroprotection in acute stroke studies, infarct volume is typically reported with a correction for brain edema.[41] This may be appropriate in mice with normal metabolism, but this study clearly shows the negative impact of obesity not only on infarct size and brain swelling but also higher mortality and increased incidence of hemorrhagic transformation. The significant correlation we observed between brain swelling and infarct size in lean, but not in obese, mice (Figure 1C) indicates that the obesity-exacerbated brain swelling is an independent pathological process that causes infarct expansion. Edema is a life-threatening complication during infarct development in patients,[13] animal studies showing that bilateral craniotomies reduce infarct size[42] further suggest that brain edema is a driving pathology influencing infarction expansion and that there is a need to develop interventions to target this process, particularly when comorbid conditions are present.

The current study demonstrated a relationship between obesity and VEGF pathway expression and function. We found the stroke in obese mice increases VEGF-A and VEGFR2 expression and is accompanied by exacerbation of brain swelling and vascular permeability. The targeted knockdown of VEGF signaling by aflibercept supports a mechanistic link between VEGF signaling and obesity-enhanced swelling. There are several additional strategies available to target VEGF signaling, which includes antibodies, decoy receptors, and inhibitors of VEGF-related downstream signal molecules. Studies that targeted VEGFR2 phosphorylation

in experimental diabetic mice showed reduced vascular permeability, brain swelling, and enhanced functional recovery in mice.[25,43] These previous reports are consistent with our demonstration that treatment of aflibercept in obese mice reduced brain edema, mortality, and BBB disruption. Our findings also clearly indicate that VEGF signaling is overactivated in obesity-comorbid stroke and support anti-VEGF intervention strategies.

The recognized model of aflibercept is by high-affinity binding to VEGF-A isotypes and thereby acting as a decoy VEGF receptor, which inhibits VEGF signaling.[32–34] Despite the fact that aflibercept acts as a decoy receptor for VEGF ligands, we unexpectedly observed reduced VEGF-A gene expression in animals treated with aflibercept. Several previous studies have reported that aflibercept treatment can decrease VEGF-A gene expression under pathological conditions.[44–46] Since VEGF/VEGFR2 signaling induces a feed-forward loop that is mediated by a VEGFR2/PI3K/mTOR/VEGF signaling cascade in lung cancer,[47] we speculate that interrupting this amplifying cascade underlies the reduced VEGF-A mRNA expression levels in our studies. Additionally, we also showed that aflibercept reduced endothelial NRP-1 expression (Figure III in the Data Supplement), a protein that interacts with VEGFR2 to promote endothelial tip cell function during angiogenesis.[38] The reduction of NRP-1 expression is further evidence that aflibercept effectively downregulated VEGF signaling in our system. The overall safety of using aflibercept to target VEGF signaling is supported by its clinical use to treat age-related macular degeneration, retinopathy of prematurity, and cancer.[32,33] Given the current lack of viable clinical options to treat acute stroke, the observed benefits shown in our current study, as well as in previous reports, support repurposing aflibercept for treatment of acute stroke in obese subjects.

Brain edema is a serious complication of large infarction that is associated with worsening clinical outcomes.[48,49] In malignant infarction, targeting SUR1 (sulfonylurea receptor 1)-TRPM4 (transient receptor potential melastatin 4)-AQP4 (aquaporin 4) complex has been suggested to reduced brain edema.[49,50] Since larger strokes are accompanied by greater brain swelling, we also tested the efficacy of aflibercept for malignant infarction in nonobese mice. Unlike when the obesity comorbidity is present, aflibercept did not provide a clear benefit in nonobese mice with large stroke. The lack of efficacy may be a consequence of lower VEGFR2 activation levels in nonobese mice compared with that of obese mice (cf. Figures 2B versus 6B). Despite the clear lack of benefit, it is also important to note that aflibercept did not worsen stroke outcomes in nonobese mice with severe stroke. Notably, the treatment showed a trend toward better survival during the first 48 hours (Figure 6D). Together, our study suggests that the mechanism for stroke-induced brain swelling is different between lean and obese mice, but it also shows that the aflibercept administration is safe in both lean and obese subjects.

Limitations to be possible clinical use of aflibercept include reported side effects, such as hypertension, neutropenia, bleeding, and hemorrhage.[51,52] The single application of 10 mg/kg aflibercept used in this study (compared with clinical dosage of 4 mg/kg every 2 weeks), however, neither caused hypertension nor changes in leukocyte number in the blood (Figure V in the Data Supplement). In addition, the reduced incidence of hemorrhagic transformation in aflibercept-treated obese mice (Figure 5B) suggests that our treatment protocol would be unlikely to cause adverse effects. Other caveats are the unknown consequences of the acute inhibition of VEGF signaling on long-term functional recovery. Early administration of VEGF at 1 hour poststroke exacerbates ischemic damage and increases hemorrhagic transformation, whereas delayed administration until 48 hours poststroke improves microvascular plasma perfusion, angiogenesis, and neurological recovery.[53] Thus, there is a disparity in the pathophysiology of VEGF interventions that is dependent on the poststroke time point (eg, acute versus repair/recovery) and metabolic status (lean versus obese). This disparity points to the critical issue of therapeutic windows for either inhibiting or activating VEGF signaling. Future studies should better define the timing window(s) for modulating VEGF signaling to not only benefit acute outcomes but also sustained functional recovery in normal and obese subjects. Besides directly targeting VEGF-A or VEGFR2, modulating heparin sulfate-mediated VEGF bioactivity induces angiogenesis and neurogenesis and enhances functional recovery after stroke.[54] In addition, Ang2 (angiopoietin-2) and Ang1/Tie2 (Tek tyrosine kinase) have been studied as targets to reduce stroke-induced brain edema.[55,56] Targeting Ang2, however, increases infarct size and vascular permeability,[54] whereas targeting Ang1 reduces brain edema and improves neurological functions.[56] These results indicate that molecules modulating vascular leakage and angiogenesis have a therapeutic potential for reducing brain edema in stroke with comorbidity.

In summary, this study demonstrates that targeting VEGF signaling by aflibercept in obese mice attenuates brain edema without affecting infarct size. The selective reduction of brain edema in obese mice is accompanied by reduced BBB disruption, hemorrhagic transformation, and behavioral impairment. Our studies suggest that obesity-enhanced VEGF signaling may represent a shift from a necessary adaptive response to hypoxia in normal metabolic conditions to a pathological event when obesity is present. This comorbidity-induced shift results in the formation of dysfunctional and leaky vessels as well as a disparity in stroke pathology between normal and metabolically

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compromised conditions. Considering the limited pharmacological options to attenuate stroke-induced brain swelling, the findings from this study support repurposing aflibercept to treat stroke in obese subjects.

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Disclosures

None.

Supplemental Materials

Expanded Materials and Methods
Online Figures I–V

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