Rapamycin induces an eNOS dependent increase in brain collateral perfusion in wistar and spontaneously hypertensive rats

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Abstract

Background and purpose—Rapamycin is a clinically approved mammalian target of rapamycin inhibitor that has been shown to be neuroprotective in animal models of stroke. However, the mechanism of rapamycin-induced neuroprotection is still being explored. Our aims were to determine if rapamycin improved leptomeningeal collateral perfusion, to determine if this is through endothelial nitric oxide synthase (eNOS)-mediated vessel dilation and to determine if rapamycin increases immediate post-reperfusion blood flow.

Methods—Wistar and spontaneously hypertensive rats (SHR) (~14 weeks old, n = 22 and n = 15, respectively) were subjected to ischemia by middle cerebral artery occlusion (MCAo, 90 and 120 min, respectively) with or without treatment with rapamycin at 30 minutes post-stroke. Changes in MCA and collateral perfusion territories were measured by dual-site laser Doppler. Reactivity to rapamycin was studied using isolated and pressurized leptomeningeal anastomoses (LMA). Brain injury was measured histologically or with triphenyltetrazolium chloride staining.

Results—In Wistar rats, rapamycin, increased collateral perfusion ($43 \pm 17\%$), increased reperfusion cerebral blood flow (CBF) ($16 \pm 8\%$) and significantly reduced infarct volume (35 ± 6 vs. $63 \pm 8 mm^3$, $p < 0.05$). Rapamycin dilated LMA by $80 \pm 9\%$, which was abolished by nitric oxide synthase inhibition. In SHRs, rapamycin increased collateral perfusion by $32 \pm 25\%$, reperfusion CBF by $44 \pm 16\%$, without reducing acute infarct volume 2 h post- reperfusion. Reperfusion CBF was a stronger predictor of brain damage than collateral perfusion in both Wistar and SHRs.

Conclusions—Rapamycin increased collateral perfusion and reperfusion CBF in both Wistar and co-morbid SHRs that appeared to be mediated by enhancing eNOS activation. These findings
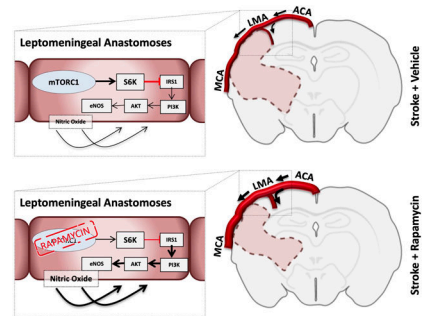
suggest that rapamycin may be an effective acute therapy for increasing collateral flow and as an adjunct therapy to thrombolysis or thrombectomy to improve reperfusion blood flow.

Graphical Abstract

During middle cerebral artery Occlusion (MCAo) in both Wistar and Spontaneously Hypertensive rats (SHR), leptomeningeal collateral perfusion was increased in a nitric oxide (NO) dependent manner that was associated with a reduction in infarct volume and improvement of neurobehavioral scores in Wistar rats but not SHR. Rapamycin also increased reperfusion cerebral blood flow (CBF) in both Wistar and SHR. Reperfusion CBF was a stronger predictor of infarct volume than collateral CBF.

Keywords

Collateral Circulation; eNOS; Experimental Stroke; mTOR; Rapamycin

Introduction

Stroke is a leading cause of death and adult disability worldwide.[1] Despite multiple drugs showing promise in preclinical studies, none have translated to clinical practice.[2] Amplifying the brain's intrinsic cytoprotective pathways is a promising avenue for developing new treatments for stroke. Mammalian Target of Rapamycin Complex 1 (mTORC1) has been shown to play a role in neuronal degeneration following global ischemia.[3] In states of sufficient energy supply, mTORC1 is activated; signaling anabolic cellular processes such as protein synthesis and cell proliferation.[4] Low cellular energy, such as in cerebral ischemia, induces TSC1 (Hamartin) and TSC2 (Tuberin), subsequently down-regulating mTORC1.[5] Pharmacological inhibition of mTORC1 with the FDA approved anti-rejection medication, rapamycin, has been shown to be a promising brain cytoprotectant in experimental models of stroke.[6] However, the mechanisms of rapamycin-induced brain cytoprotection are still be being explored.[7]

The success of revascularisation therapy in stroke has led stroke researchers to reconsider the important role of the cerebral perfusion in acute stroke therapy.[8] Rapamycin has been shown to improve cerebral blood flow (CBF) in Alzheimer's Disease (AD) mouse models via increased activation of endothelial nitric oxide synthase (eNOS).[9] In the context of ischemic stroke, the degree of perfusion through leptomeningeal collateral anastomoses (LMAs) is one of the strongest predictors of penumbral volume, eligibility for

revascularization therapies and ultimately stroke outcome.[10] Unfortunately, not all patients have adequate collateral perfusion and these patients progress to infarction rapidly and may be ineligible for revascularization therapy or not benefit if they receive this therapy.[11]

Collateral therapeutics is an emerging area of investigation to determine if interventions can increase collateral flow in the acute setting and potentially improve patient eligibility for revascularization therapy and improve stroke outcome.[12] Hypertension is the leading modifiable risk factor for stroke[13] and is associated with poor LMA blood flow and worse stroke outcomes in human[14] and animal stroke.[15] LMAs from hypertensive animals have been shown to be more vasoconstricted than normotensive LMAs,[16] thus they have more space to dilate and may benefit more from collateral enhancement therapy. Therefore, it is imperative that potential collateral therapies show efficacy in both normal and hypertensive animals.

Given the evidence of rapamycin's cerebrovascular effect in other neurological diseases we wanted to determine whether leptomeningeal collateral perfusion was affected by rapamycin, the potential contribution of the eNOS pathway to this process, and the effects of rapamycin on reperfusion CBF in normotensive and hypertensive rats.

Methods

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Animals

Wistar rats (male, 12–14 weeks old, n = 22) and Spontaneously Hypertensive Rats (SHR, male, 12–14 weeks old, n = 15) were used in this study. All procedures conformed to the Animal (Scientific Procedures) Act 1986 (UK), the National Institutes of Health guidelines for care and use of laboratory animals and were approved by the University of Oxford Animal Ethics Committee, the Home Office (UK) and the Institutional Animal Care and Use Committee at the University of Vermont. The studies were conducted and the manuscript prepared in accordance with the ARRIVE guidelines.[17] Detailed animal and experimental descriptions are available in the online-only Data Supplement, please see https:// www.ahajournals.org/journal/str.

Model of Transient Focal Ischemia and Treatment

A proximal middle cerebral artery occlusion (MCAo) filament model was used. Rats underwent MCAo using the silicone-tipped intraluminal thread occlusion method, as previously described.[18, 19]

Multisite Laser Doppler Flowmetry (LDF)

Multisite laser Doppler probes (Oxford Optronix, Oxford, UK) were used to measure changes in the MCA and collateral (LMA) perfusion territories, as described previously (Figure 1A).[20] Probe locations are based off cerebrovascular casting studies[21, 22] and have been validated using magnetic resonance imaging[23] and hydrogen clearance.[24]

MCAo was confirmed by >70% decrease in LDF signal from baseline in Probe 1. We selected > 70% drop from baseline CBF based on the infarct threshold of 20–30% of baseline CBF previously reported in normotensive and hypertensive rats.[25, 26] In Wistar rats, LMA (probe 2) and MCA (probe 1) perfusion were recorded throughout the whole experiment. Changes in LMA perfusion are expressed as a % of pre-rapamycin administration at 30 min post-MCAo (baseline). Animals were randomized by sealed numbered envelope to receive vehicle (<5% ethanol in saline, n = 5) or rapamycin (Sigma Aldrich, 250 μg/kg, n=8), administered by a surgeon blind to treatment allocation. LMA recordings continued until thread withdrawal (reperfusion) at 90 min post-MCAo and reperfusion core-MCA territory CBF (probe 1, from here on referred to as reperfusion CBF) was monitored for a further 40 min.

Following 24 hours of recovery, the adhesive removal test was used to assess somatosensory deficits following stroke.[27] Animals were also tested for stroke-induced neurologic deficits (methods in online-only Data Supplement).[28] Brains were then taken for infarct volume analysis (Supplementary Figure I A).

In SHRs, administration of rapamycin, and recordings of LMA (probe 2) and MCA (probe 1) perfusion were conducted using the same protocol as Wistar rats, except LMA recordings continued until reperfusion at 120 min post-MCAo and reperfusion CBF was monitored for a further 120 min. SHR have more severe strokes, which can have a negative impact on animal welfare during recovery. As the primary outcome of this study was collateral perfusion, which could be assessed during the stroke under anesthesia these experiments were non-recovery with acute infarct volume assessment. Brains were taken for infarct volume analysis at 120 min post-reperfusion (Supplementary Figure I B).

Histological Analysis of Brain Damage

In Wistar rats, infarct volume was calculated at 24 h after stroke onset using coronal sections stained with hematoxylin and eosin.[29] In SHRs, infarct volume was determined by 2,3,5-triphenyltetrazolium chloride (TTC) staining at 4 h post MCAo. Volumes were then corrected for oedema using the formula: corrected infarct volume (mm^3) = infarct volume x (contralateral volume/ ipsilateral volume).[30] LDF and histological analyses were performed by investigators blind to treatment allocation.

Reactivity of Isolated LMAs

Reactivity of isolated LMAs to rapamycin was determined using the method previously in Chan et al.[16, 31] Detailed description of this method and calculation of % tone and % reactivity of isolated LMAs can be found in in the online-only Data Supplement, please see https://www.ahajournals.org/journal/str

Excluded Animals

Wistar: Four animals were excluded from vehicle-treated group: 2 due to insufficient LDF drop during filament insertion, 1 due to filament-induced haemorrhage and 1 due to lack of final infarct. *SHR*: Three animals were excluded: 1 rapamycin and 2 vehicle, due to

experimental complications. *Isolated LMAs*: One animal was excluded because of insufficient tone development.

Statistical Analysis

Statistical tests were performed using Graphpad Prism 7.0 (La Jolla, USA). D'Agostino and Pearson omnibus normality tests were performed on all data. Appropriate statistical tests were used based on the normality of the data. Detailed description of statistical tests and sample size calculations for LMA perfusion are available in the online-only Data Supplement. Data are presented as mean ± standard error of the mean (SEM).

Results

Multi-site LDF probes were measuring different hemodynamic regions

MCAo was confirmed by a 70% reduction in MCA perfusion in LDF probe 1 (core/MCA). Figure 1 A and B shows that the drop in peri-infarct/LMA perfusion (probe 2) was significantly less than core flow (probe 1), demonstrating that the two probes were measuring different hemodynamic regions in both Wistar and SHR, respectively. Values are available in the online-only Data Supplement.

Rapamycin improved LMA perfusion, reperfusion CBF, infarct volume and neurological deficits in Wistar rats

Rapamycin significantly increased LMA perfusion (collateral flow) during MCAo (Probe 2) at 10 min post-drug administration, relative to vehicle and immediate pre-injection baseline (rapamycin: 43.4 ± 17.4 vs. vehicle: -15.6 ± 9.9 % of pre-injection baseline, $p < 0.05$, Figure 1D), demonstrating rapamycin treatment increased collateral flow. Rapamycin significantly increased reperfusion CBF (Probe 1). Reperfusion CBF was increased at 35 min, relative to vehicle and immediate post-reperfusion baseline (rapamycin: 16.2 ± 8.4 vs. vehicle: $-20.9 \pm 13.6\%$ of immediate post-reperfusion baseline, $p < 0.01$, Figure 2A).

Rapamycin significantly reduced infarct volume at 24 h (rapamycin: 35.7 ± 5.6 mm^3 vs. vehicle: 62.7 ± 7.9 mm^3, $p < 0.05$, Figure 2B and C). There was significantly less neurological deficit at 24 h in animals treated with rapamycin (time-to-touch on contralateral forepaw = 37.8 ± 18.0 s in the rapamycin group vs. 113.3 ± 39.0 s in the vehicle group, $p < 0.05$). There was no significant change in time-to-touch on the ipsilateral forepaw between 0 and 24 h in either group (Figure 2D). Rapamycin showed a trend towards improved neuroscores (Rapamycin: median score of 2, range 1–3 vs. Vehicle: median score of 4, range 2–5 out of a max score of 6, Supplementary Figure II).

Rapamycin improved LMA perfusion and reperfusion CBF but not early infarct volume in SHRs

There were no significant differences in physiological variables in SHRs (mean arterial pressure, pO_2, pCO_2 and pH) between the rapamycin and vehicle groups prior to injection, 10 min after treatment and 60 min post-reperfusion (Table 1).

Rapamycin significantly increased LMA perfusion (collateral flow) during MCAo (Probe 2) relative to pre-injection baseline at 45, 75 and 80 min post-drug administration (80 min: 31.8 ± 24.7 % pre-injection baseline, $p < 0.01$, Figure 3A). Rapamycin significantly increased reperfusion CBF (Probe 1). Reperfusion CBF was increased relative to vehicle at 70 min post-reperfusion (rapamycin: 43.7 ± 16.1 vs. vehicle: - 7.2 ± 9.2 % of post-reperfusion baseline, $p < 0.05$, Figure 3B). Rapamycin also significantly increased reperfusion CBF relative to baseline between 5 and 85 min post-reperfusion (65 min: 47.5 ± 13.4 % post-reperfusion baseline, $p < 0.0001$, Figure 3B). Rapamycin did not reduce final infarct volume (rapamycin: 76.3 ± 11.2 mm^3 vs. vehicle: 76.4 ± 16 mm^3, $p > 0.05$, Figure 3C and D).

Reperfusion CBF is a stronger predictor of outcome than LMA perfusion in Wistar and SHR

In Wistar rats, average LMA perfusion during experimental stroke was not significantly correlated with final infarct volume ($r^2 = 0.007$, $p > 0.05$, Figure 4A). Average reperfusion CBF was significantly inversely correlated with final infarct volume ($r^2 = 0.4298$, $p < 0.05$, Figure 4B).

In SHRs, average LMA perfusion during experimental stroke was not significantly correlated with final infarct volume ($r^2 = 0.08$, $p > 0.05$, Figure 4C). Average reperfusion CBF was not-significantly inversely correlated with final infarct volume ($r^2 = 0.1$, $p > 0.05$, Figure 4D).

Rapamycin dilation of isolated collateral vessels is dependent on NOS

Isolated LMA active lumen diameters were 35 ± 5 μm and % tone was 15.2 ± 3.4% at 40mm Hg. Rapamycin caused a dose-dependent vasodilation of LMAs (Figure 5 A and C). Percent reactivity to 10^{-6} mol/L rapamycin was 79.7 ± 9.4 % and 7.3 ± 3.6% in the presence of L-NAME, respectively ($p < 0.01$, Figure 5 B and C).

Discussion

Improving collateral perfusion during vessel occlusion and microcirculatory perfusion following vessel re-opening are attractive therapeutic targets to improve penumbral survival and stroke outcome. In the current study we found that the clinically approved mTORC1 inhibitor, rapamycin, improved collateral perfusion in both normotensive Wistar rats and hypertensive SHRs, the latter being known to have poor baseline collateral perfusion. Rapamycin appeared to enhance collateral perfusion by an eNOS-dependent mechanism, as demonstrated in isolated and pressurized LMAs. We also found that rapamycin improved reperfusion CBF immediately after stroke in Wistar rats and SHRs and that this was a stronger predictor of final infarct volume stroke outcome. We have also shown that rapamycin reduced upper limb somatosensory impairments in rodents. This is important from a translational perspective, as homologous upper limb somatosensory impairments are also common in stroke patients.

Rapamycin significantly increased collateral perfusion in Wistar rats. Our results are in agreement with those of Wang et al.[32], who showed that intraperitoneal administration of 10mg/kg of rapamycin immediately after MCAo in Sprague Dawley rats significantly

increased the diameter of deep brain collaterals between the terminal branches of the posterior cerebral artery and MCA. They also reported that the same dose and timing of rapamycin administration in C57BL/6 mice resulted in a modest but significant improvement MCA territory perfusion between 10 and 60 min after MCAo, measured by Laser Speckle Contrast Imaging. Although these findings provide evidence of rapamycin enhancing blood flow to the core MCA territory via deep brain collaterals, we have shown that rapamycin also enhances perfusion to the peri-infarct territory via superficial leptomeningeal collaterals between the ACA and MCA. It is tempting to conclude that rapamycin enhanced penumbral perfusion and limited the size of the core infarction, however we did not measure penumbral volume or its progression over time. The improved infarct volume and neurological function seen at 24 hours may have been a combination of improved penumbral perfusion as well as rapamycin's many other reported brain cytoprotective effects, including but not limited to, induction of autophagy, anti-inflammatory effects and reduced blood brain barrier disruption.[7]

It is estimated that over 50% of stroke patients have hypertension.[33] Hypertension is associated with poor collateral blood flow and worse stroke outcomes in human[14] and animal models of stroke.[15] Therefore, hypertensive patients may have the most to gain from collateral therapeutics, if these vessels were amenable to therapies. Early animal studies revealed that SHRs have smaller diameter collateral vessels than normotensive Wistar Kyoto rats.[21] This was thought to be due to remodeling of the vessels due to hypertension[34] and that these vessels were unlikely to respond to acute therapeutic intervention. However, recent studies by the Cipolla group have demonstrated that LMAs from SHRs have increased vasoconstriction, such as significantly higher myogenic tone, compared to LMAs from normotensive Wistar Kyoto rats and that hypertensive collateral vessels dilate to various vasoactive mediators.[16, 20, 31] This indicates greater vasoconstriction (not remodeling) of collateral vessels compared to normotensive animals, and that they are amenable to pharmacological vasodilation.

Our results further confirm the ability to enhance collateral perfusion in hypertensive animals, as rapamycin caused a variable but significant increase in collateral perfusion in SHR. The variability of SHR collateral perfusion and smaller effect size compared to Wistar collateral perfusion was surprising, given that SHR collaterals have greater tone under physiological intravascular pressure,[16] thus we anticipated SHR collaterals having greater potential to dilate in response to rapamycin. It is possible that SHR collaterals were less responsive to nitric oxide (NO)-mediated vasodilation, which may explain this discrepancy. [16] Rapamycin may have increased eNOS activation and NO production in the endothelium, as suggested by our Wistar results; however, the smooth muscle cells of the LMAs from young SHR may have been less responsive to NO, as has been shown previously.[18] Interestingly, SHR LMAs appear to regain their sensitivity to NO during ageing[16] and therefore rapamycin may be more effective in enhancing LMA in aged SHR. However, determination of the effect of rapamycin on LMA perfusion in aged SHR and the reactivity of young and aged isolated SHR LMA to rapamycin was beyond the scope of the current study and will be the focus of future work and publications.

We did, however, show that rapamycin dilated isolated collaterals from Wistar rats and the non-specific NOS inhibitor L-NAME blocked this dilation. The role of NOS inhibition in stroke is controversial, with NOS inhibitors showing conflicting effects on infarct volume and cerebral blood flow.[35] This may be due to the lack of NOS isoform specificity of drugs such L-NAME. Targeted disruption of eNOS *in vivo* (eNOS knock-out mice) has shown the importance of eNOS in maintaining collateral perfusion during stroke.[36] An advantage of our approach of utilizing L-NAME in isolated vessels, rather than *in vivo*, is that the isolated vessels are removed from the brain and are free from peri-vascular innervation (intrinsic innervation) and thus neuronal NOS.[16] Therefore, the inhibitory effect of L-NAME on rapamycin induced dilation strongly suggest that rapamycin dilates collateral vessels via activation of eNOS rather than any other NOS isoforms.

This is in line with previous studies showing that rapamycin can increase eNOS phosphorylation and expression in mouse aorta[37] and can relax aortic rings.[38] Rapamycin has also been shown to increase eNOS expression in areas of low shear stress in carotid arteries of mice,[39] as well as increase eNOS expression and phosphorylation in the brains of human amyloid precursor protein (AD) mice, leading to cortical vessel dilation, improved CBF and improved cognitive function in these animals.[9] A possible mechanism for rapamycin-induced activation of eNOS is by rapamycin reducing mTORC1's feedback inhibition of AKT, thus allowing AKT to phosphorylate eNOS and increase NO production.[39]

Structural and functional differences in pial collateral circulations have been studied in both male and female adult Wistar rats. It has been shown that LMAs from both sexes had similar myogenic tone and reactivity to pressure, similar vascular function, and similar numbers of LMAs.[40] However, it is unknown if there are sex differences in collateral function during chronic hypertension. We recognize that the use of only one sex is a limitation of the current study; however, inclusion of both males and females with treatment was outside the scope of the current study. Future studies will determine the generalizability of our findings to female normotensive and hypertensive rats as well as aged animals.

In the current study, vehicle treated animals showed a decline in reperfusion CBF in the core-MCA territory in both Wistar and SHR, which has been reported in a number of previous studies.[20, 24, 31, 41, 42] We show that rapamycin treatment prevented the decline in reperfusion CBF. We are aware of only one previous *in vivo* stroke study that has measured reperfusion CBF following rapamycin treatment. Although this study reported a non-significant increase in CBF at 2 hours post reperfusion in male Fischer 344 rats, this was associated with an increase in infarct volume measured at the same time.[43] This discrepancy may be explained by the chronic (three days) high-dose administration (20 mg/kg) of rapamycin used in this study, which may have led to toxic off target effects in other neurovascular cells.[6, 7]

Decline in reperfusion CBF has been attributed to pericyte or smooth muscle constriction following ischemia.[44, 45] Rapamycin may be reducing pericyte constriction through a Rho-A dependent pathway.[46] Alternatively, rapamycin may be enhancing eNOS phosphorylation/activity and thus increasing smooth muscle cell relaxation on penetrating arterioles[47] or

directly reduce calcium sensitivity in smooth muscle cells to reduce vasoconstriction of penetrating arterioles.[44] Direct evidence of either mechanism is yet to be obtained and warrants further investigation.

Unlike previous studies, we did not show a significant relationship between LMA perfusion and brain injury in either Wistar rats and SHRs.[20, 23] However, we found that the degree of reperfusion CBF was significantly inversely correlated with final infarct volume in Wistar rats and was a stronger predictor of outcome compared to collateral perfusion in SHR. This is in line with previous studies that have shown that reperfusion CBF is a significant predictor of stroke outcome.[41, 42] Our findings strongly suggest that rapamycin may be useful being administered to prevent impaired microvascular perfusion or the so-called "no-reflow" phenomenon in patients eligible for thrombolysis or thrombectomy.

Conclusions

Rapamycin significantly enhanced collateral perfusion and reperfusion CBF during experimental stroke. Isolated collateral experiments demonstrated that this increase in perfusion was potentially driven by rapamycin dilating collaterals. Rapamycin's vasodilatory effect was largely inhibited by L-NAME, suggesting that rapamycin dilated LMAs through a mechanism involving eNOS. Rapamycin also improved MCA perfusion following stroke and this was a stronger predictor of stroke outcome. Therefore, our findings suggest that rapamycin may be an effective acute collateral enhancing therapy as well as an intervention to improve reperfusion CBF to improve stroke outcome.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements:

Non-standard Abbreviations and Acronyms

|	
AD	Alzheimer's Disease
CBF	Cerebral Blood Flow
eNOS	Endothelial Nitric Oxide Synthase
LMA	Leptomeningeal Anastomosis

L-NAME	Nω-nitro-L-arginine methyl ester
MCAo	Middle Cerebral Artery Occlusion
mTORC1	Mammalian Target of Rapamycin Complex 1
NO	Nitric Oxide
SHR	Spontaneously Hypertensive Rat
TSC 1	Tuberosclerosis 1 (hamartin)
TSC 2	Tuberosclerosis 2 (tuberin)
TTC	2,3,5-triphenyltetrazolium chloride

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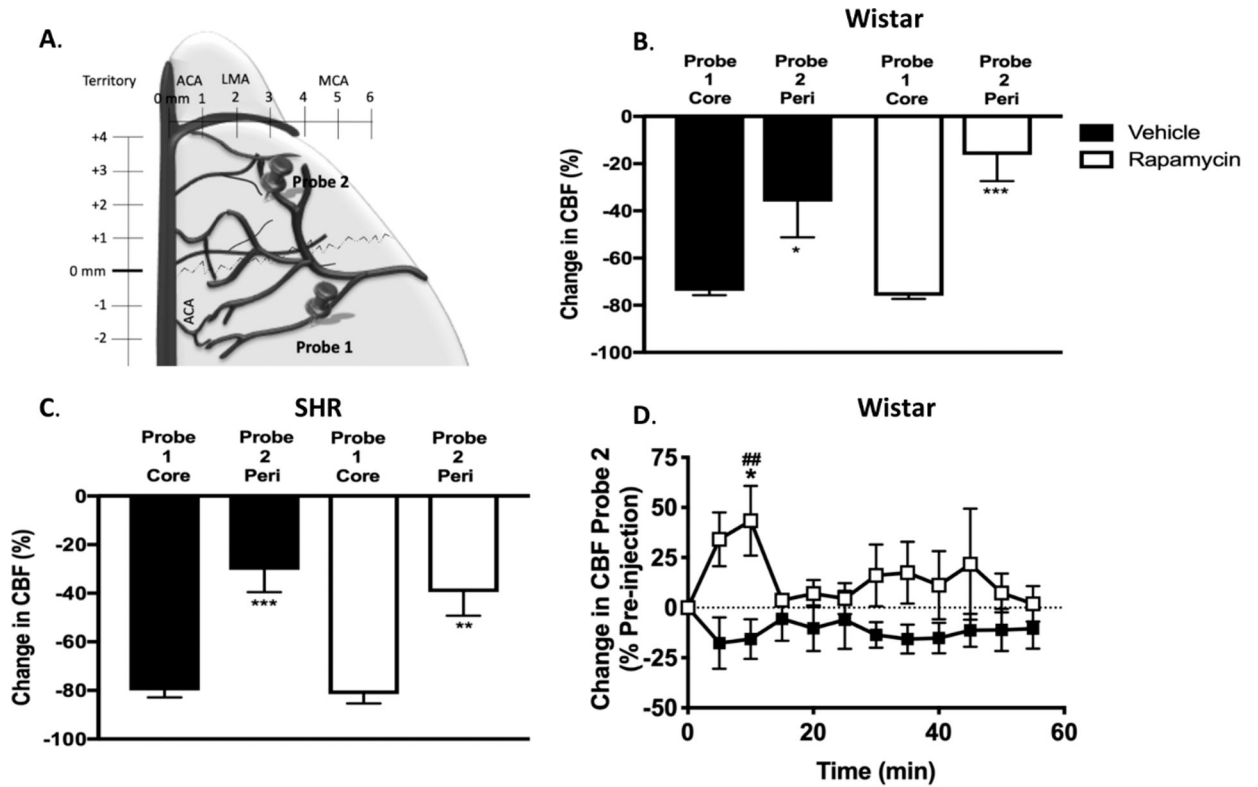
**Figure 1. Multisite laser Doppler Flowmetry for measurement of collateral perfusion.**
A. Schematic showing the location of the dual-site laser Doppler probes. B and C. Graphs comparing initial drop in cerebral blood flow (CBF) from baseline before filament insertion between MCA core (Probe 1) and peri-infarct (Probe 2) collateral CBF in Wistar rats and SHRs, respectively. Drop in collateral CBF (probe 2) was significantly less than that of the core MCA territory (probe 1) demonstrating that each probe was measuring different hemodynamic areas. *p < 0.05, **p < 0.01, *** p < 0.001 vs. probe 1 group, unpaired t-tests. D. Change in Leptomeningeal Anastomoses (LMA) CBF in Wistar rats calculated as % change from prior to treatment (30min after middle cerebral artery occlusion (MCAo). RM 2-Way ANOVA: F (1, 11) = 3.429, p = 0.09 for treatment, F (11, 121) = 0.5054, p > 0.05 for time, F (11, 121) = 1.463, p > 0.05 for interaction. *p < 0.05 vs. vehicle, Sidak's post-test. ## p < 0.01 vs. pre-injection baseline in rapamycin group, Dunnett's post-test.
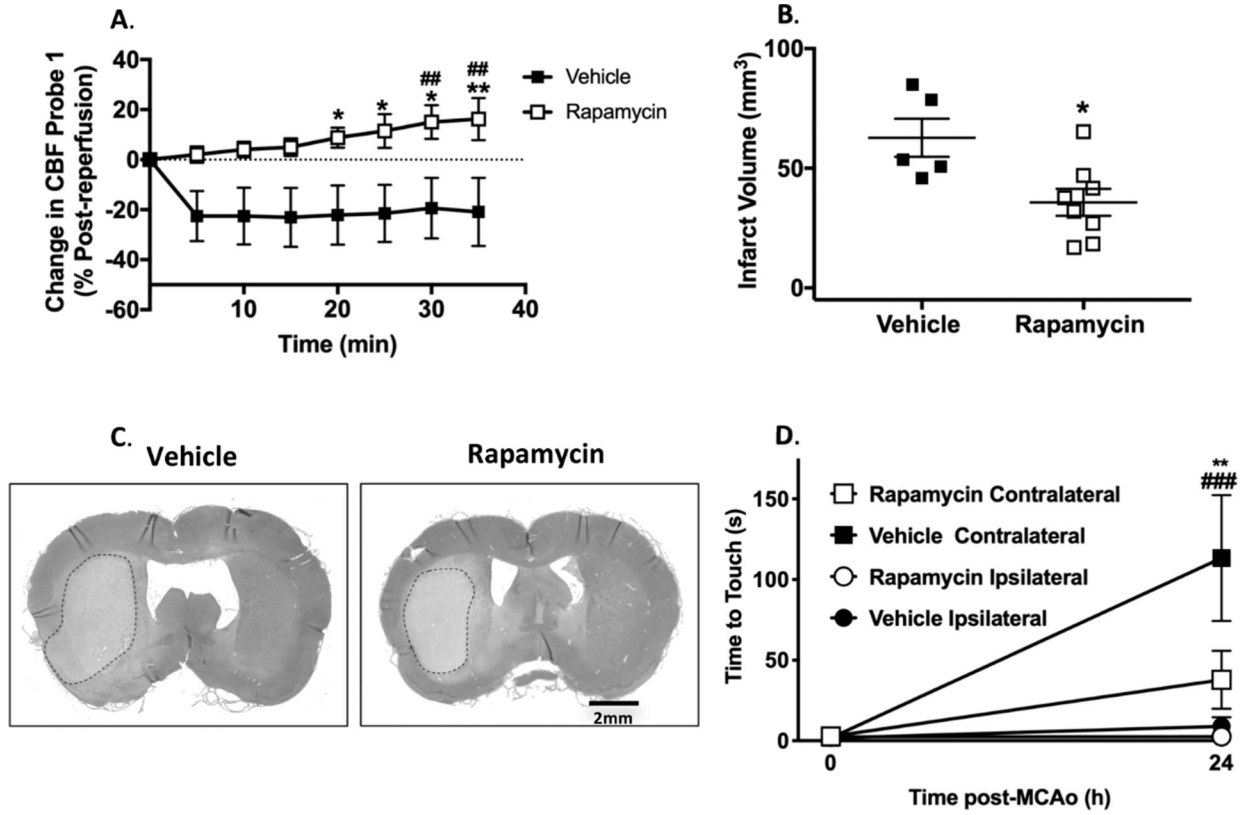
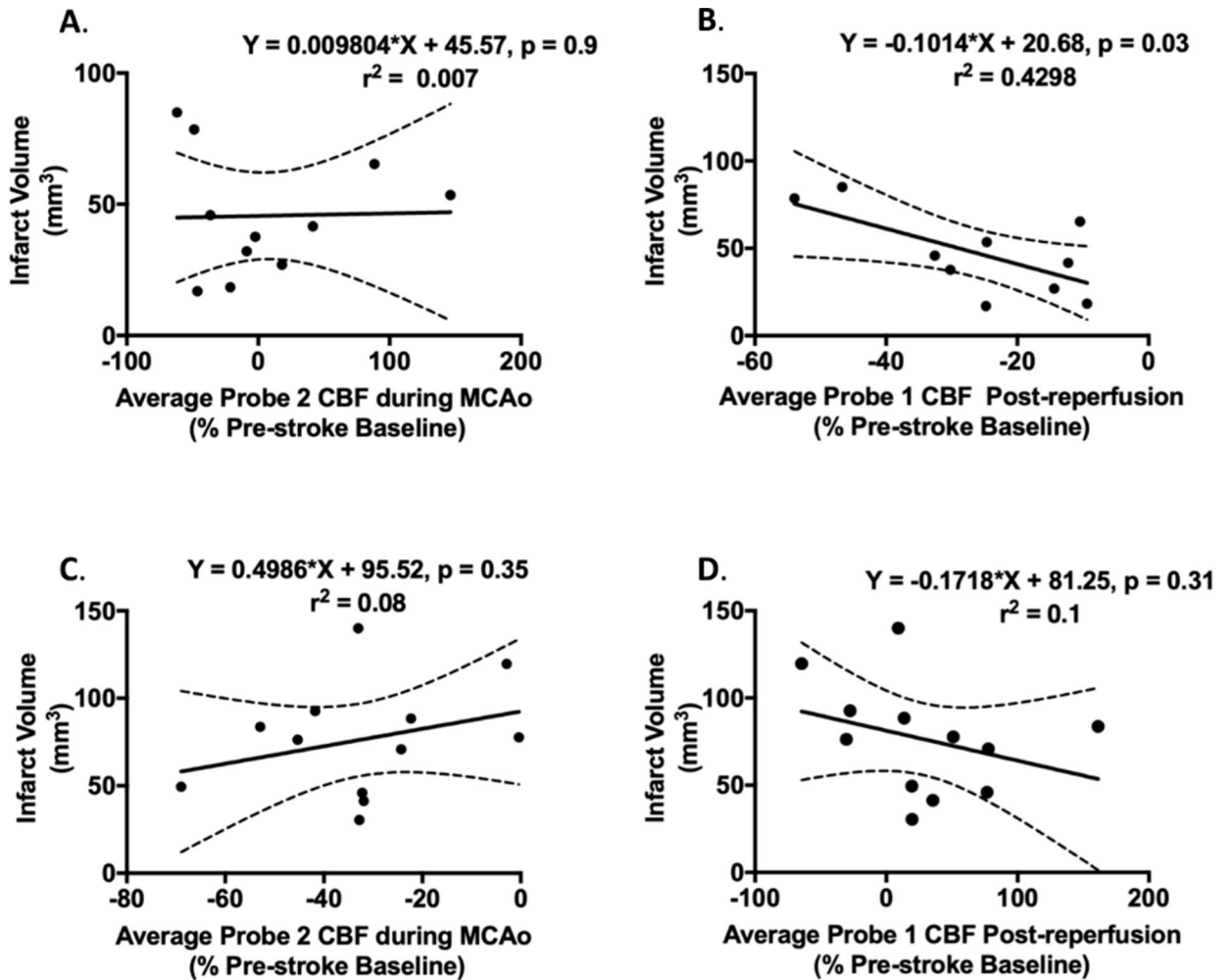**Figure 2. Wistar rats reperfusion cerebral blood flow and stroke outcome analysis.**
A. Change in middle cerebral artery (MCA) cerebral blood flow calculated as % change post- reperfusion (95 min post-MCAo). RM 2-Way ANOVA: $F_{(1, 64)} = 49.11$, $p < 0.0001$ for treatment, $F_{(7, 64)} = 0.4979$, $p = 0.83$ for time, $F_{(7, 64)} = 1.15$, $p = 0.35$ for interaction. $*p < 0.05$, $** p < 0.01$ vs. vehicle, Sidak's post-test. ## $p < 0.01$ vs. post-reperfusion baseline in rapamycin group, Dunnett's post-test. B. Infarct volume at 24 h post-MCAo. $*p < 0.05$ vs. vehicle, unpaired t-test. C. Representative histological hematoxylin and eosin stains for infarct volume. D. Time to touch in adhesive removal test at pre-MCAo baseline and 24 h after MCAo. RM 2-Way ANOVA: $F_{(3, 22)} = 6.253$, $p < 0.001$ for treatment, $F_{(1, 22)} = 16.32$, $p < 0.01$ for time, $F_{(3, 22)} = 6.375$, $p < 0.01$ for interaction. $**p < 0.01$ rapamycin contralateral vs. vehicle contralateral, Tukey's post-test. ### $p < 0.001$ vs. pre-MCAo baseline in vehicle group, Sidak's post-test.
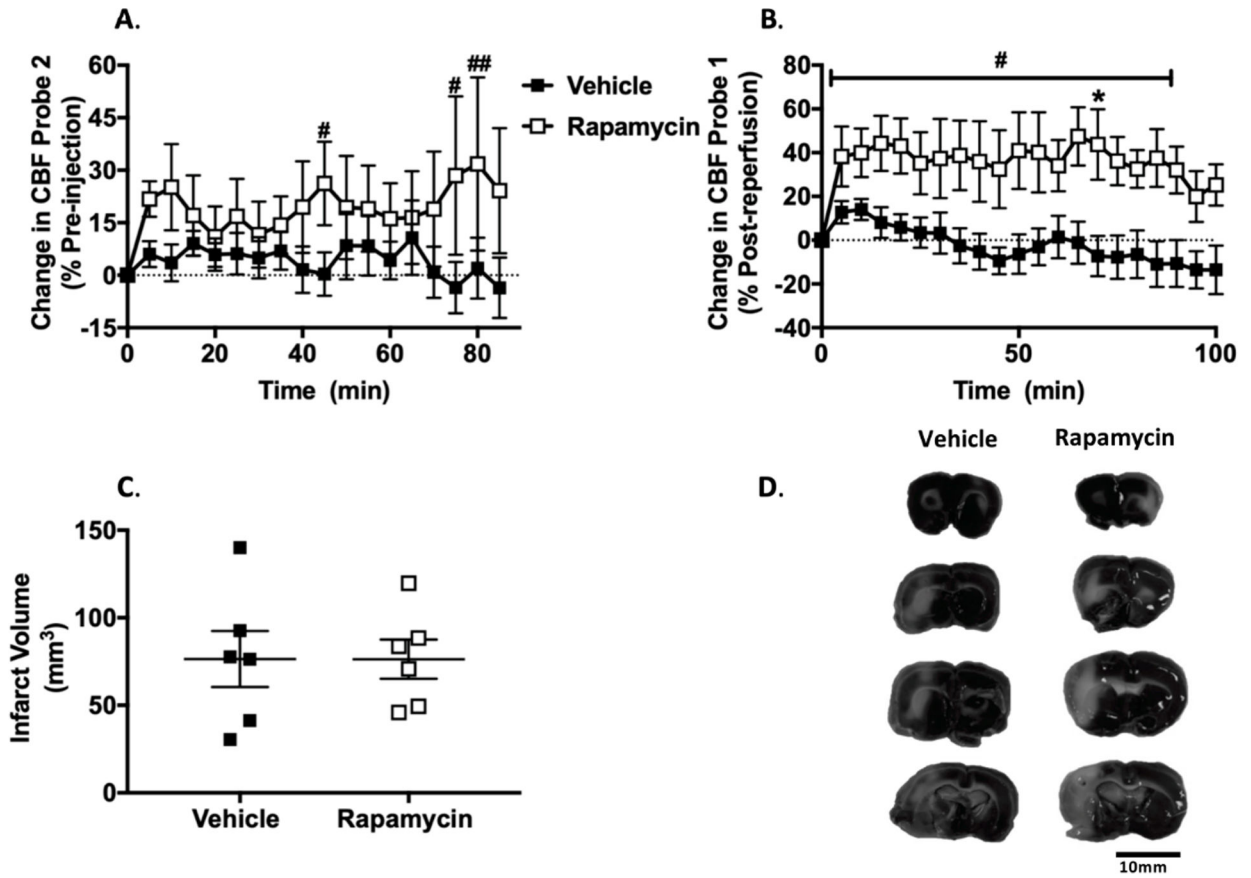
Figure 3. Spontaneously Hypertensive Rats (SHRs) studies.

A. Change in leptomeningeal anastomoses cerebral blood flow (CBF) calculated as % change from prior to treatment at 30min after middle cerebral artery occlusion (MCAo). RM 2-Way ANOVA: $F_{(1, 10)} = 1.403$, $p > 0.05$ for treatment, $F_{(17, 170)} = 0.6585$, $p > 0.05$ for time, $F_{(17, 170)} = 1.102$, $p > 0.05$ for interaction. # $p < 0.05$, ## $p < 0.01$ vs. pre-injection baseline in rapamycin group, Dunnett's post-test. B. Change in MCA CBF calculated as % change from reperfusion (125 min post-MCAo). RM 2-Way ANOVA: $F_{(1, 10)} = 8.433$, $p < 0.05$ for treatment, $F_{(20, 200)} = 2.113$, $p < 0.01$ for time, $F_{(20, 200)} = 1.202$, $p > 0.05$ for interaction.* $p < 0.05$ vs. vehicle, RM 2-Way ANOVA, Sidak's post-test. # $p < 0.05$ vs. post-reperfusion baseline in rapamycin group, RM 2-Way ANOVA, Dunnett's post-test. C. Infarct volume at 4 h post-MCAo. D. Representative TTC stains.

Figure 4. Relationship between changes in leptomeningeal anastomoses (LMA) cerebral blood flow (CBF), reperfusion CBF and final infarct volume.

A-B. Wistar rats. A. Correlation between average LMA perfusion during middle cerebral artery occlusion (MCAo) and 24 hour infarct volume. There was a non-significant inverse correlation found between LMA blood flow and final infarct volume. B. Correlation between average reperfusion CBF and 24 hour infarct volume. There was a significant inverse correlation found between reperfusion CBF and infarct volume. C-D. SHRs. C. Correlation between average LMA perfusion during MCAo and 4 hour infarct volume. There was no significant correlation between LMA CBF and final infarct volume in SHRs. D. Correlation between Average reperfusion CBF and 4 hour infarct volume. There was no significant correlation between reperfusion CBF and infarct volume in SHRs.
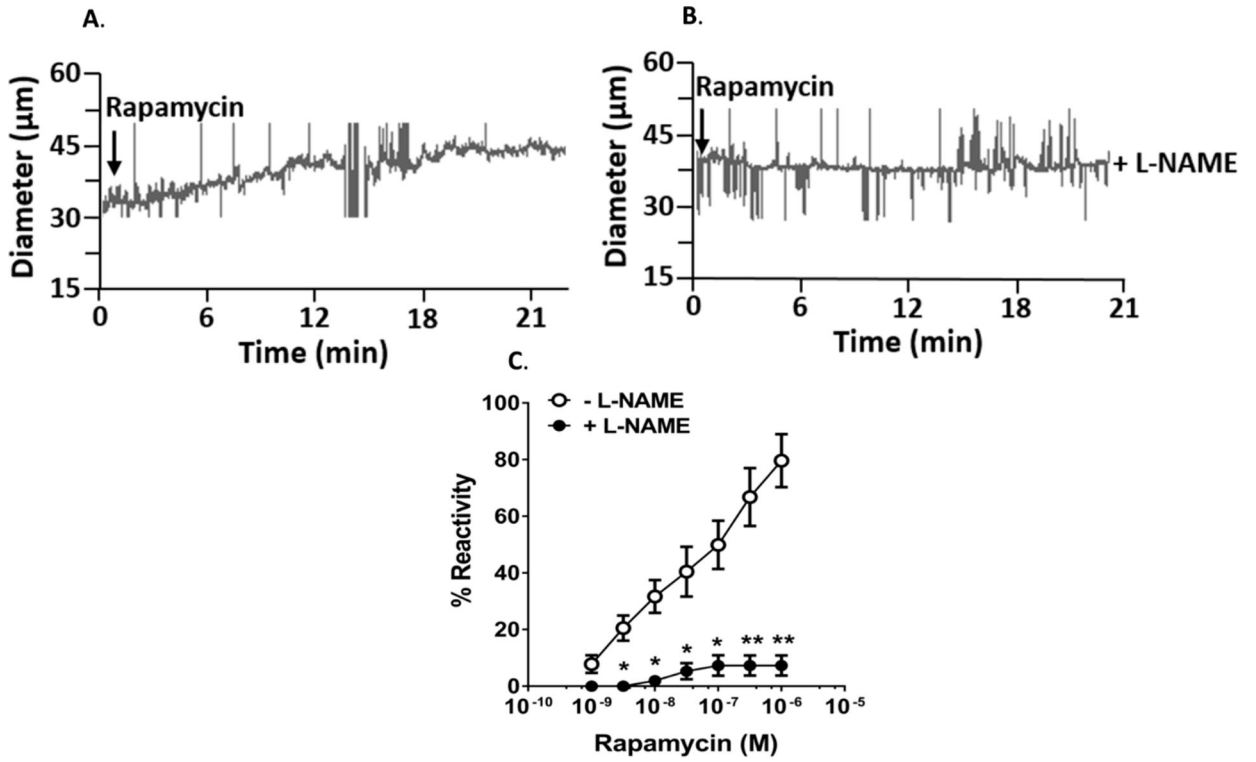
Figure 5. Isolated leptomeningeal anastomoses (LMAs) from Wistar rats.
A and B. Representative tracing of changes in diameter of isolated and pressurized LMAs in response to rapamycin and rapamycin in the presence of L-NAME, respectively. C. Graph showing percent reactivity of isolated LMA to rapamycin in the absence and presence of L-NAME. Addition of rapamycin caused concentration-dependent vasodilation that was prevented by nitric oxide inhibition with L-NAME. * $p < 0.05$, **$p < 0.001$ vs. LMAs without L-NAME treatment.

Table 1.

Physiological Parameters

SHR	Pre-injection		10min post-injection		Reperfusion	
	Vehicle	Rapamycin	Vehicle	Rapamycin	Vehicle	Rapamycin
MAP (mm Hg)	177 ± 8.2	180.3 ± 7.3	174.5 ± 9.6	184.5 ± 4.5	134.8 ± 4.4	141.5 ± 7.9
pO2 (mm Hg)	128.4 ± 5.4	140.4 ± 3.1	131.2 ± 4	135 ± 3.9	135.9 ± 2.5	140.4 ± 4.7
pCO2 (mm Hg)	38.4 ± 1.4	39.7 ± 1.5	38.2 ± 1.1	39.5 ± 2.2	40.8 ± 3.3	47.3 ± 0.4
pH	7.44 ± 0.008	7.43 ± 0.01	7.41 ± 0.01	7.38 ± 0.008	7.35 ± 0.005	7.34 ± 0.007

Abbreviations: MAP, mean arterial pressure; pCO2, partial pressure of carbon dioxide; pO2, partial pressure of oxygen.