Post-ischaemic silencing of p66Shc reduces ischaemia/reperfusion brain injury and its expression correlates to clinical outcome in stroke

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Abstract: Aim: Constitutive genetic deletion of the adaptor protein p66Shc was shown to protect from ischaemia/reperfusion injury. Here, we aimed at understanding the molecular mechanisms underlying this effect in stroke and studied p66Shc gene regulation in human ischaemic stroke. Methods and Results: Ischaemia/reperfusion brain injury was induced by performing a transient middle cerebral artery occlusion surgery on wild-type mice. After the ischaemic episode and upon reperfusion, small interfering RNA targeting p66Shc was injected intravenously. We observed that post-ischaemic p66Shc knockdown preserved blood-brain barrier integrity that resulted in improved stroke outcome, as identified by smaller lesion volumes, decreased neurological deficits, and increased survival. Experiments on primary human brain microvascular endothelial cells demonstrated that silencing of the adaptor protein p66Shc preserves claudin-5 protein levels during hypoxia/reoxygenation by reducing nicotinamide adenine dinucleotide phosphate oxidase activity and reactive oxygen species production. Further, we found that in peripheral blood monocytes of acute ischaemic stroke patients p66Shc gene expression is transiently increased and that this increase correlates with short-term neurological outcome. Conclusion: Post-ischaemic silencing of p66Shc upon reperfusion improves stroke outcome in mice while the expression of p66Shc gene correlates with short-term outcome in patients with ischaemic stroke.

DOI: https://doi.org/10.1093/eurheartj/ehv140
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Word count of manuscript: 7’151

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Abstract

Aim Constitutive genetic deletion of the adaptor protein p66\(^{Shc}\) was shown to protect from ischaemia/reperfusion injury. Here, we aimed at understanding the molecular mechanisms underlying this effect in stroke and studied p66\(^{Shc}\) gene regulation in human ischaemic stroke.

Methods and Results Ischaemia/reperfusion brain injury was induced by performing a transient middle cerebral artery occlusion surgery on wild-type mice. After the ischaemic episode and upon reperfusion, small interfering RNA targeting p66\(^{Shc}\) was injected intravenously. We observed that post-ischaemic p66\(^{Shc}\) knockdown preserved blood-brain barrier integrity that resulted in improved stroke outcome, as identified by smaller lesion volumes, decreased neurological deficits, and increased survival. Experiments on primary human brain microvascular endothelial cells demonstrated that silencing of the adaptor protein p66\(^{Shc}\) preserves claudin-5 protein levels during hypoxia/reoxygenation by reducing nicotinamide adenine dinucleotide phosphate oxidase activity and reactive oxygen species production. Further, we found that in peripheral blood monocytes of acute ischaemic stroke patients p66\(^{Shc}\) gene expression is transiently increased and that this increase correlates with short-term neurological outcome.

Conclusion Post-ischaemic silencing of p66\(^{Shc}\) upon reperfusion improves stroke outcome in mice while the expression of p66\(^{Shc}\) gene correlates with short-term outcome in patients with ischaemic stroke.

Key words: stroke, ischaemia, reperfusion, free radicals, endothelium
Translational Perspective

In light of the limited repertoire of therapeutical options available for the treatment of ischaemic stroke, the identification of novel potential targets is vital; in this respect, the present study demonstrates that the adaptor protein p66Shc holds this potential as an adjunct therapy to thrombolysis. Post-ischaemic silencing of p66Shc protein yielded beneficial effects in a mouse model of ischaemia/reperfusion brain injury underlying an interesting translational perspective for this target protein. Further, in proof-of-principle clinical experiments using peripheral blood monocytes we demonstrate that p66Shc gene expression is transiently increased and that its levels correlate to short-term outcome in ischaemic stroke patients. Although these latter experiments are not directly relevant to the experiments performed in mice and in human endothelial cells, they provide novel important information about p66Shc regulation in stroke patients and set the basis for further investigations aimed at assessing the potential for p66Shc to become a novel therapeutic target as an adjunct of thrombolysis for the management of acute ischaemic stroke.
Introduction

Stroke is associated with major disabilities and mortality. Although over the last decades several novel experimental neuroprotective strategies have been developed, their translation into clinical practice has proven difficult. Thus, the search for novel therapeutic targets for ischaemic stroke as an adjunct to thrombolysis remains an unmet clinical need.

Although ischaemic stroke is amenable to thrombolysis in patients presenting early after symptom onset, vascular leakage and the ensuing oedema formation during reperfusion contributes importantly to neurological deficits. Cerebral microvascular endothelial cells are a main component of the blood-brain barrier (BBB) which divides the cerebral circulation from brain tissue. These cells are interconnected by tight and adherens junction proteins whose integrity is critical for stroke outcome. Indeed, disruption of the BBB following ischaemia/reperfusion (I/R) leads to vascular leakage and infiltration of plasma components into the brain tissue leading to oedema and further organ damage. Overproduction of reactive oxygen species (ROS) following I/R is considered a key mechanism leading to BBB damage. p66Shc, an isoform of the mammalian adaptor protein Shc, is a crucial mediator of ROS production in several disease states thereby leading to cellular apoptosis. Indeed, much of the vasculoprotective properties observed by genetic deletion of p66Shc in mice are the result of reduced oxidative stress and in turn preserved endothelial function.

In line with the above, we previously demonstrated that mice lacking p66Shc develop smaller stroke size following I/R. However, the clinical relevance of this observation remains unknown and the underlying molecular mechanisms poorly understood. To this end, we subjected mice to I/R brain injury and, to mimic real life clinical conditions as it would occur in the context of thrombolysis, we performed p66Shc silencing in vivo using small interfering RNA (siRNA) after the ischaemic episode and upon reperfusion. Additionally, we mimicked I/R conditions in primary human brain microvascular endothelial cells (HBMEC) by exposing them to hypoxia/reoxygenation (H/R) with and without silencing of p66Shc and assessed the production of ROS as well as the levels of tight and adherens junction proteins. Lastly, we studied p66Shc gene expression in peripheral blood monocytes (PBM) of patients with acute ischaemic stroke and correlated it to the National Institutes of Health Stroke Scale (NIHSS).
Methods

Patients

Twenty-seven patients admitted to the emergency room of San Raffaele Hospital (OSR, Milan, Italy) with a diagnosis of acute ischaemic stroke presenting within 6 h from symptom onset were enrolled. Five patients presented wake-up stroke and were recruited within 6 h from awakening. The initial diagnosis was based on clinical history, neurological examination (conducted by certified neurologists) and a brain computed tomography (CT) scan. Nineteen sex- and age-matched healthy volunteers (either relatives or visitors of in-hospital patients), with a negative history of cardio- and cerebrovascular diseases, were included as controls. Patients diagnosed with diabetes, systemic inflammatory diseases, acute infections, and malignancy were excluded, to eliminate potential interference of those disease states on p66\textsuperscript{Shc} expression\textsuperscript{23}. Blood was withdrawn from the antecubital vein at 6 h and 24 h after initial stroke symptoms (for stroke patients), whereas control subjects donated blood once.

Out of the 27 ischaemic stroke patients, 14 received thrombolytic treatment within 4.5 h from initial stroke symptoms onset. Ischaemic strokes were clinically classified according to the Oxford Community Stroke Project classification (OCSP, also known as the Bamford or OXFORD classification)\textsuperscript{24}. Stroke aetiology was classified according to the Trial of ORG 10172 in Acute Stroke Treatment (TOAST) criteria\textsuperscript{25}. Moreover, stroke severity was assessed, using NIHSS on admission and at discharge. Furthermore, delta NIHSS was calculated as the difference between the NIHSS presented at discharge and the NIHSS presented at admission (delta NIHSS = NIHSS discharge – NIHSS admission); thereby, positive values indicate short-term neurologic worsening while negative values indicate neurological improvement.

The study was approved by the local Ethics Committee at San Raffaele Scientific Institute, Milan, Italy. All participants (or their representative relatives) signed a written informed consent to authorise the treatment of their biological and clinical data.

Isolation of peripheral blood monocytes

PBM from whole-blood were isolated using anti-CD14-coated MicroBeads (Miltenyi Biotec) on a magnetic separator (Miltenyi Biotec), as previously described\textsuperscript{26}.
Animals

All animal experiments were performed on male, 11-13 weeks old wild-type (wt) (C57/BL6J) mice. Study design and experimental protocols were approved by the Cantonal Veterinary Office of the Canton of Zurich.

Middle cerebral artery occlusion (MCAO)

A transient middle cerebral artery (MCA) occlusion (MCAO) surgery was performed on wt mice to induce I/R brain injury, as described\textsuperscript{22}. In brief, anaesthesia was induced with 3% isoflurane in oxygen-enriched air and mice were kept under 1.5% isoflurane anaesthesia during MCAO surgery. Body temperature was controlled by using a warm water heating pad. Incision site was infiltrated with 0.5% bupivacaine solution for pain relief. A 6-0 silicone-coated filament (Doccol Corporation) was advanced into the internal carotid artery (ICA) until the thread occluded the origin of the left MCA to induce unilateral MCAO. After 45 min (60 min for evaluation of long-term effect) of ischaemia, the thread was removed to allow reperfusion of the MCA. After wound care and before returning to their standard cage, mice were kept for 1.5 h in a temperature controlled cage. For sham operation, the filament was advanced into the ICA without occluding the MCA.

In vivo p66\textsuperscript{Shc} silencing

In vivo p66\textsuperscript{Shc} silencing was performed as described\textsuperscript{27}. Briefly, 1.6 nmol of predesigned siRNA targeting p66\textsuperscript{Shc} were incubated with a mixture of 150 mmol/L NaCl solution–jetPEI\textsuperscript{®} and injected intravenously into the tail vein of the wt mouse randomized. Scrambled siRNA was used as a negative control. Detailed methods are provided in the supplemental material.

Magnetic resonance imaging (MRI)

Lesion development was monitored after MCAO on a Bruker PharmaScan 47/16 (Bruker BioSpin GmbH) operating at 4.7 T. Anaesthesia was induced using 3% isoflurane (Abbott) in a 4:1 air/oxygen mixture. During MRI acquisition, mice were kept under isoflurane anaesthesia (1.5%). During the scan session body temperature was monitored with a rectal temperature probe (MLT415, ADInstruments) and kept at 36±0.55°C
using a warm water circuit integrated into the animal support (Bruker BioSpin GmbH). MRI recordings were done in a blinded way by an independent person.

The lesion was determined on maps of the apparent diffusion coefficient (ADC) derived from diffusion-weighted images as areas of significant reduction of the ADC compared to the unaffected, contralateral side. The lesion in the T2-weighted image was determined as hyperintense areas compared to the contralateral hemisphere. Lesion volumes were quantified blinded by drawing region of interests around the areas of reduced ADC and hyperintensities in T2-weighted images in five MRI slices using a ROI tool (Paravision, Bruker). Brain infarct volumes were calculated by summing the volumes of each section and correcting for brain swelling, as described\textsuperscript{28}. Detailed methods are provided in the supplemental material.

**Neurological deficits measurement**

Neurological status was assessed using an adapted 4-point scale test based on Bederson \textit{et al.},\textsuperscript{29} and was graded as previously described\textsuperscript{22}: grade 0: normal neurological function; grade 1: forelimb flexion; grade 2: circling; grade 3: leaning to the contralateral side at rest; grade 4: no spontaneous motor activity. Motor performance was assessed using the RotaRod test. Mice were placed on a rotating rod with increasing speed (4 – 44 revolutions/min; circumference of the rod: 9 cm) and the latency to fall was measured. The experimental trial was ended if the maximum rotation speed was achieved, or if the mouse fell off the rod. Per time point, 3 test runs per mouse were performed and the best run was included in the statistics. Neurological deficit measurements were performed in a blinded way.

**Evaluation of long-term effect**

Long-term effect of post-ischaemic \textit{in vivo} p66\textsuperscript{Shc} silencing on stroke was assessed up to 6 days. The well-being of mice during the experimental period was determined using a score sheet that was approved by the Cantonal Veterinary Office of the Canton of Zurich. This score sheet was used to define survival/death of an animal. Death events include spontaneous deaths (4 out of 16 for siScr stroke mice and 3 out of 15 for sip66\textsuperscript{Shc} stroke mice) and mice which did not fulfil the health evaluation criteria.
Evans blue extravasation

Determination of BBB permeability after MCAO was done by evaluating Evans blue extravasation, as described\textsuperscript{30}. Detailed methods are provided in the supplemental material.

Immunofluorescence staining

Frozen brains were cut into 6 µm thick slices on a cryostat (Leica Cm 1900). Immunofluorescent analysis was performed as described\textsuperscript{31}. Briefly, brain slices were fixed in 4% paraformaldehyde and incubated with primary and secondary antibodies. Images were taken using a Leica Dm4000 B microscope. Stained area of claudin-5, vascular endothelial (VE)-cadherin, or occludin was measured using ImageJ Software and normalised to the total endothelial cell surface (assessed by isolectin B\textsubscript{4} staining). Detailed methods are provided in the supplemental material.

RNA isolation and reverse transcription

Total RNA isolation and preparation of cDNA was performed as previously described\textsuperscript{26}. Total RNA of PBM, or of MCA, was extracted using TRIZol Reagent (Invitrogen). Two µg (MCA), or 1 µg (PBM) of RNA was reverse transcribed using Ready-To-Go You-Prime First-Strand Beads (Amersham Biosciences) and first-strand random cDNA primers pd(N)6 (TaKaRa).

Quantitative real-time PCR

Determination of p66\textsuperscript{Shc} gene expression was done as previously described\textsuperscript{26}. Detailed methods are provided in the supplemental material.

Cell culture experiments

Primary human brain microvascular endothelial cells (HBMEC; Cell Systems) were cultured in EBM-2 medium supplied with EGM-2 bullet kit (Lonza). Adhering cells (passages 5 to 9) were grown to confluence and exposed to hypoxia (0.2% oxygen) for 4 h, followed or not by 4 h of incubation in a normoxic incubator
Hypoxia was induced using a gas-controlled glove box (Invivo2 400, Ruskinn Technologies). In certain experiments, cells were pre-incubated with apocynin (0.1 mmol/L) (SAFC) for 1 h. Thereafter, HBMEC were harvested for measuring superoxide anion (O$_2^-$) production, nitric oxide (NO) bioavailability, nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activity or immunoblot analysis.

**siRNA transfection in HBMEC**

HBMEC were incubated with predesigned siRNA targeting p66$^{Shc}$ at final concentration of 25 nmol/L using Lipofectamine®RNAiMAX Reagent (Invitrogen), as previously described$^{32}$. Detailed methods are provided in the supplemental material.

**Immunoblotting**

Basilar arteries and cells were lysed in lysis buffer and proteins were separated using SDS-PAGE, as previously described$^{32}$. Detailed methods are provided in the supplemental material.

**Measurement of O$_2^-$ production and NO bioavailability**

Electron spin resonance (ESR) spectroscopy was applied to determine O$_2^-$ production and NO bioavailability, as described$^{27,32-34}$. Detailed methods are provided in the supplemental material.

**NADPH oxidase activity**

NADPH oxidase activity was determined indirectly by measuring the ratio of NADP/NADPH using a commercially available kit (Abcam), according to the manufacturer’s recommendations.

**Statistical analysis**

Statistical analysis for comparison of 2 groups was performed using 2-tailed unpaired Student’s $t$-test, or Mann-Whitney test, when appropriate. For comparison of more than 2 unmatched groups, 1-way analysis of
variance (ANOVA) followed by Bonferroni post hoc test, or Kruskal-Wallis test followed by Dunn’s post hoc test, when appropriate, was performed. For comparison of groups with repeated measures, 2-way ANOVA followed by Bonferroni post hoc test was applied. Statistical analysis for survival studies was performed using Log-rank (Mantel-Cox) test. Pearson’s correlation analysis was used to test the correlation between two quantitative variables, and Fisher’s exact test for comparison of categorical data between study subjects. Two-sided P-values were calculated and P<0.05 denoted a significant difference. Statistical analysis was performed using GraphPad Prism® software 5.01.
Results

*In vivo* post-ischaemic silencing of p66<sup>Shc</sup> reduces lesion volumes and improves functional outcome following I/R brain injury

To study the effect of post-ischaemic p66<sup>Shc</sup> silencing on stroke, a transient MCAO surgery was performed on wt mice to induce I/R brain injury. To analyse p66<sup>Shc</sup> silencing efficiency *in vivo* beforehand, p66<sup>Shc</sup> mRNA and protein levels were quantified in cerebral arteries (MCA, and basilar artery, respectively). Intravenous injection of siRNA against p66<sup>Shc</sup> reduced mRNA and protein p66<sup>Shc</sup> levels within 48 h after injection compared to siScr injection (Figure 1A–B). To localise the distribution of the p66<sup>Shc</sup> siRNA within brain cerebral arteries, wt mice were injected with fluorescence dye-labelled p66<sup>Shc</sup> siRNA (Alexa546-sip66<sup>Shc</sup>). Flow cytometry with whole-brain digests revealed a predominant uptake of the siRNA by the brain endothelium. We observed that 21.2% of brain endothelial cells (CD45<sup>−</sup>CD31<sup>+</sup>) showed a positive signal for Alexa546-tagged p66<sup>Shc</sup> siRNA (Figure 1F) while only 0.6% of leukocytes (CD45<sup>+</sup>CD31<sup>−</sup>) (Figure 1G) and 0.2% of other nucleated cells (CD45<sup>−</sup>CD31<sup>−</sup>) (Figure 1H) were positive for the Alexa546 signal. Negative control was stained with Hoechst 33342 to label nucleated cells (Figure 1L–N).

Lesion volumes in sip66<sup>Shc</sup> and siScr injected stroke mice were quantified with MRI. DWI denoted matching baseline lesion sizes in both groups after 45 min of ischaemia and directly upon reperfusion (Figure 2B). At 48 h post-MCAO both DWI and T2-weighted imaging displayed instead a reduced stroke volume in sip66<sup>Shc</sup> stroke mice as compared to siScr stroke mice (Figure 2B–C).

Neurological deficits after MCAO were assessed using two different tests. Pre-MCAO, both sip66<sup>Shc</sup> and siScr stroke mice showed comparable performance in the RotaRod test and on the 4-point scale test based on Bederson *et al.*<sup>29</sup> (Figure 2D–E). At 24 h post-MCAO we observed in both groups a reduced latency to fall in the RotaRod test. However, at 48 h post-MCAO, sip66<sup>Shc</sup> stroke mice showed a significant higher persistence on the rotating drum compared to siScr stroke mice (Figure 2D). In line with that, neurological deficits assessed with the scale test were significantly lower in sip66<sup>Shc</sup> stroke mice compared to siScr stroke mice at 48 h post-MCAO (Figure 2E). All sham operated animas displayed normal neurological functions throughout the experimental period (Figure 2D and data not shown).
By analysing the impact of p66\textsuperscript{Shc} silencing on stroke up to 6 days, we found an improved survival of sip66\textsuperscript{Shc} stroke mice as compared to siScr stroke mice (Figure 2F, 31.25% survival for siScr stroke mice vs. 66.66% for sip66\textsuperscript{Shc} stroke mice). Survival was assessed in accordance to the health evaluation criteria approved by the Cantonal Veterinary Office of the Canton of Zürich; all sham operated animals survived and fulfilled the health evaluation criteria (data not shown).

**In vivo p66\textsuperscript{Shc} silencing preserves BBB integrity after I/R brain injury**

The BBB plays a critical role for the outcome of stroke\textsuperscript{8}, and its permeability can be assessed *in vivo* by quantifying Evans blue extravasation\textsuperscript{9, 30}. We tested whether post-ischaemic p66\textsuperscript{Shc} silencing preserves BBB permeability after I/R brain injury in mice. Indeed, 48 h post-MCAO p66\textsuperscript{Shc} silencing reduced Evans blue extravasation as compared to mice receiving siScr (Figure 3A).

BBB permeability is regulated by tight and adherens junctional proteins connecting cerebral microvascular endothelial cells\textsuperscript{35}. Thus, we analysed the integrity of tight and adherens junctions following I/R brain injury focusing on claudin-5, occludin, and VE-cadherin. Immunofluorescence staining of claudin-5 on coronal brain sections of siScr-treated stroke mice revealed decreased claudin-5-positive stained areas normalised to the total endothelial surface (measured by isolectin B\textsubscript{4} staining\textsuperscript{31, 36}) in the ipsilateral hemisphere compared to the contralateral hemisphere (Figure 3B), while this disruption of claudin-5 integrity was not observed in stroke mice receiving sip66\textsuperscript{Shc} (Figure 3B). Unlike claudin-5, occludin and VE-cadherin-positive stained areas were not changed following I/R brain injury in both experimental groups (data not shown).

**Role of p66\textsuperscript{Shc} in H/R in primary HBMEC**

In order to characterise the molecular regulation of claudin-5 by p66\textsuperscript{Shc} and to translate our *in vivo* murine data to human cells, we exposed HBMEC to hypoxia (H) alone, or hypoxia followed by reoxygenation (H/R). Exposure of HBMEC to hypoxia neither altered phosphorylation of p66\textsuperscript{Shc} at Ser36, a critical step for its pro-apoptotic\textsuperscript{37} and pro-oxidant activity\textsuperscript{21}, nor total p66\textsuperscript{Shc} protein levels significantly, compared to normoxia
(Figure 4A). By contrast, exposure to H/R increased phosphorylation of p66Shc at Ser36 compared to normoxia (Figure 4B). Hypoxia-inducible factor-1 alpha (HIF-1α) protein stabilisation was used as an indicator of effective hypoxic conditions (Figure 4A). ROS and NO are critical for endothelial function\textsuperscript{30}, and have been implicated in vascular permeability\textsuperscript{12}. Here, we analysed whether H/R regulates O\textsubscript{2}\textsuperscript{−} production and NO bioavailability, and whether p66\textsuperscript{Shc} mediates these effects. Exposure of HBMEC to H/R led to an increased O\textsubscript{2}\textsuperscript{−} production (Figure 4D) and a decreased NO bioavailability (Figure 4E) compared to normoxia. However, endothelial NO synthase (eNOS) phosphorylation at both sites studied (Ser1177 and Thr495) and eNOS protein expression remained unchanged (Figure 4F–G). Pre-incubation of HBMEC with p66\textsuperscript{Shc} siRNA, but not scrambled siRNA, reduced the increased O\textsubscript{2}\textsuperscript{−} production (Figure 4D) and increased the reduced NO bioavailability during H/R (Figure 4E). NADPH oxidase is considered as a major source of vascular O\textsubscript{2}\textsuperscript{−}\textsuperscript{38}, and is a downstream target of p66\textsuperscript{Shc}\textsuperscript{34, 39}. Moreover, NADPH oxidase was demonstrated to play a dominant role in ROS production in brain endothelial cells exposed to H/R\textsuperscript{40}. Thus, we investigated whether during H/R NADPH oxidase is a source of O\textsubscript{2}\textsuperscript{−} production downstream of p66\textsuperscript{Shc}. Indeed, NADPH oxidase activity was increased after exposure to H/R, and returned to control levels after silencing of p66\textsuperscript{Shc} (Figure 4H).

**Effect of p66\textsuperscript{Shc} and ROS on claudin-5 in vitro**

HBMEC exposed to H/R exhibited reduced protein levels of claudin-5 compared to normoxia (Figure 5A). In contrast, occludin and VE-cadherin protein levels were not affected by exposure to H/R (Figure 5B–C). After both, pre-incubation of HBMEC with p66\textsuperscript{Shc} siRNA and with the antioxidant apocynin\textsuperscript{41} claudin-5 levels were elevated under H/R condition (Figure 5D–E).

**p66\textsuperscript{Shc} gene expression is increased in patients with ischaemic stroke and correlates to neurological outcome**

To provide evidence for a role of p66\textsuperscript{Shc} in human ischemic stroke, we analysed p66\textsuperscript{Shc} expression in PBM of patients with ischaemic stroke. A total of 27 ischaemic stroke patients and 19 age- and sex-matched, healthy control subjects were recruited. Clinical characteristics of both groups did not differ statistically (Table 1).
p66\textsuperscript{Shc} mRNA levels were significantly increased 6 h after initial stroke symptoms (Figure 6A) and returned to control levels 24 h thereafter (Figure 6A). Of note, p66\textsuperscript{Shc} gene expression at 6 h was comparable in patients with ischaemic stroke regardless of whether they had received thrombolytic treatment or not (Figure 6B). p66\textsuperscript{Shc} transcript levels of all study subjects positively correlated with neurological deficits at admission, measured according to the NIHSS (Figure 6C). Importantly, while p66\textsuperscript{Shc} gene expression of stroke patients which did not receive thrombolytic intervention did not correlate with short-term neurological outcome (NIHSS discharge – NIHSS admission) (Figure 6D), it positively correlated in stroke patients treated with thrombolysis (Figure 6E).
Discussion

In this study we demonstrate for the first time that post-ischemic p66Shc silencing reduces brain injury by preserving BBB integrity by preventing claudin-5 level downregulation. The in vivo findings in the mouse were translated to human brain microvascular endothelial cells (HBMEC) exposed to H/R where p66Shc was phosphorylated at Ser36 leading to the reduction in claudin-5 levels via activation of the NADPH oxidase and increased ROS production. Further we show that p66Shc expression is increased in PBM of patients with ischaemic stroke within 6 h from onset of symptoms and that p66Shc gene expression correlates to short-term neurological outcome.

In mice, we recently showed that constitutive genetic deletion of p66Shc reduces early stroke size and neurological deficits following I/R brain injury. However, the mechanisms of this effect and its clinical relevance remained elusive. Furthermore, the use of constitutive knockout animals only serves as a proof-of-principle and does not allow delineation of therapeutic time windows as required in the clinical setting. In contrast, RNA interference (RNAi)-based strategies allow to modify the expression of a certain target at the post-transcriptional level, thus making it therapeutically interesting. Indeed, several RNAi-based strategies are under investigation in clinical trials. Here, we used a clinically relevant experimental setup with siRNA delivery upon reperfusion to reduce p66Shc levels. Reperfusion of an occluded vessel allows reoxygenation of the ischaemic area which promotes recovery of penumbral areas. Nevertheless paradoxically, re-introduction of oxygen in a previously ischaemic area also causes a surge in free radical generation which interferes with the recovery processes. Indeed, we demonstrate here that silencing of the pro-oxidant p66Shc after the ischaemic episode and upon reperfusion, as it would be the case in stroke patients presenting at the emergency room undergoing thrombolytic treatment, reduces lesion volume, improves neurological function and increases survival. These data highlight the potential of p66Shc as novel therapeutic target in stroke patients undergoing thrombolysis.

Given the key role of BBB permeability in determining stroke outcome, we characterised its integrity by analysing Evans blue extravasation, a known indicator for vascular leakage. Indeed, and in line with the improved stroke outcome in the sip66Shc stroke mice, BBB disruption was blunted after p66Shc silencing compared to control mice. Cerebral microvascular endothelial cells connected via tight and adherens
junctional proteins make up the BBB\textsuperscript{7}. A disruption of this tightly regulated structure is known to occur in I/R brain injury and is responsible for vascular leakage and in turn oedema formation\textsuperscript{6,11}. To investigate the molecular mechanisms by which p66\textsuperscript{Shc} preserves BBB integrity after I/R brain injury, we focused \textit{in vivo} as well as \textit{in vitro} on claudin-5, occludin and VE-cadherin, three major junctional proteins\textsuperscript{7}. Consistently, we found less reduction in claudin-5 levels after silencing of p66\textsuperscript{Shc}. In contrast, occludin and VE-cadherin levels remained unaltered in both experimental settings. Our data obtained on murine as well as primary human cells indicate that p66\textsuperscript{Shc} mediates BBB disruption by acting specifically on claudin-5 rather than occludin and VE-cadherin.

To characterise the molecular regulation of claudin-5 by p66\textsuperscript{Shc}, we exposed primary human brain microvascular endothelial cells (HBMEC) to H/R, to mimic \textit{in vivo} settings. Exposure of HBMEC to H/R, but not to hypoxia, increased phosphorylation of p66\textsuperscript{Shc} at Ser36 confirming previous data in renal tubular epithelial cells\textsuperscript{46}. Together with our \textit{in vivo} data these results suggest that p66\textsuperscript{Shc} is mainly involved in mediating its deleterious effects during reperfusion, rather than during ischaemia. Endothelial ROS production and NO bioavailability are both critical for I/R-induced alteration in BBB permeability and stroke outcome\textsuperscript{12,47} and ROS are known to influence claudin-5 levels in brain endothelial cells\textsuperscript{48}. Here, we demonstrate \textit{in vitro} evidence that endothelial p66\textsuperscript{Shc} silencing in H/R preserves NO bioavailability reduces NADPH oxidase activation and decreases ROS generation thus blunting the reduction in claudin-5 levels. This pathway may be also relevant \textit{in vivo}.

To study p66\textsuperscript{Shc} gene regulation in stroke patients, we performed proof-of-principle experiments assessing its expression in peripheral blood monocytes (PBM) of acute ischaemic stroke patients. Although it would be of particular interest and relevance to elucidate the role of cerebrovascular p66\textsuperscript{Shc} in stroke patients, sample collection in humans would prove extremely difficult thus, we selected PBM since those are easily obtainable from whole-blood and could still give us interesting insights into gene expression changes. Here we found that p66\textsuperscript{Shc} mRNA levels were increased 6 h after initial stroke symptoms and then returned to basal levels at 24 h. Moreover, p66\textsuperscript{Shc} expression at 6 h correlated to short-term neurological outcome (delta NIHSS) in stroke patients. Interestingly, delta NIHSS correlated to p66\textsuperscript{Shc} expression only in patients undergoing thrombolysis, but not in those without. Although thrombolysis is known to improve neurological outcome after stroke\textsuperscript{5}, it is
also associated with early reperfusion-induced BBB damage\textsuperscript{49} which is known to be mediated by ROS and affects stroke outcome\textsuperscript{6}. An increased vascular leakage also favours the accumulation of blood circulating cells in brain tissue thus causing tissue damage via production of free radicals\textsuperscript{11, 50}. This could explain why the correlation between neurological outcome and p66\textsuperscript{Shc} expression is found only in thrombolytic patients where monocytic p66\textsuperscript{Shc}-induced ROS production and the consequent damage is likely more pronounced. In contrast, in patients not undergoing thrombolysis, the role of reperfusion-induced ROS-dependent injury is less prominent and thus neurological damage is less likely to be dependent on p66\textsuperscript{Shc}-mediated ROS.

In summary, the present study sets the stage for follow up clinical studies aimed at assessing the potential for p66\textsuperscript{Shc} to become a novel therapeutic target as an adjunct of thrombolysis for the management of acute ischaemic stroke.
**Funding**

This work was supported by the Swiss National Science Foundation [grant number 310030_147017 to GGC, 310030-135815 to T.F.L. and 136822 to JK] and Helmut-Horten Foundation to GGC.

**Acknowledgements**

We thank Prof. Gianvito Martino from the Neuroimmunology Unit, Division of Neuroscience, Institute of Experimental Neurology, San Raffaele Scientific Institute Milan, Italy, for his intellectual and technical contribution.

**Conflict of Interest**

None declared.
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Figure Legends

**Figure 1** In vivo silencing of p66Shc. (A) Quantitative real-time PCR reveals reduced p66Shc levels in middle cerebral artery homogenates 24 h (n=5-6) and 48 h (n=7-8) after p66Shc siRNA injection compared to scrambled siRNA injection (siScr). Data are expressed as mean ± s.e.m. * P<0.05 for sip66Shc vs. siScr. (B) Immunoblot analysis confirms silencing of p66Shc in basilar arteries within 48 h after p66Shc siRNA injection (representative immunoblot of at least 5 animals per group). (C–N) Flow cytometry analysis of brain single cell suspensions 24 h after injection of Alexa546-sip66Shc (upper panel) as compared to negative control (lower panel). Singlets (C; I) with nuclei (D; J) were plotted in a dot plot of APC (CD45) versus AlexaFluor488 (CD31) to distinguish endothelial cells (CD45+CD31+), leukocytes (CD45+CD31-), as well as other cell types (CD45-CD31-) (E; K). Histograms show Alexa546-fluorescence representing content of Alexa546-sip66Shc in endothelial cells (F; L), leukocytes (G; M) and other cell types (H; N).

**Figure 2** Impact of post-ischaemic in vivo p66Shc silencing on stroke outcome. (A) Schematic of experimental study design. Upon reperfusion, specific siRNA against p66Shc is injected intravenously in wt mice, and development of lesion and functional deficits were characterised. (B–C) Both, DWI and T2-weighted imaging denote reduced lesions in sip66Shc stroke mice (DWI: n=5; T2: n=6) compared to siScr stroke mice (n=7) at 48 h post-MCAO. (D–E) Evaluation of neurological deficits by using the RotaRod test and a 4-point scale test based on Bederson et al. Both neurological tests demonstrate less neurological deficits in the sip66Shc stroke group (n=9) compared to the siScr stroke group (RotaRod: n=7; Bederson: n=8). All sham operated animals show normal neurological function throughout the experimental period (n=5-6). (F) Post-ischemic silencing of p66Shc increases survival of mice after stroke (n=15-16). Data are expressed as mean ± s.e.m. * P<0.05, ** P<0.01 for sip66Shc stroke vs. siScr stroke.

**Figure 3** p66Shc mediates BBB disruption after I/R brain injury. (A) Assessment of BBB impairment. p66Shc silenced mice (n=6) show less Evans blue extravasation compared to siScr stroke mice (n=5) at 48 h of reperfusion. (B) Representative fluorescence microscopy images of claudin-5 and isolectin B4 (endothelial marker) stained brain sections. Following I/R brain injury, claudin-5-positive stained area normalised to the
total endothelial surface is reduced in the ipsilateral hemisphere compared to the contralateral hemisphere in siScr stroke mice (n=5), but not in sip66\textsuperscript{Shc} stroke mice (n=5). Scale bar, 35 µm. Data are presented as mean ± s.e.m. * P<0.05 for siScr stroke ipsilateral vs. siScr stroke contralateral; ** P<0.01 for sip66\textsuperscript{Shc} stroke vs. siScr stroke.

**Figure 4** p66\textsuperscript{Shc} mediates H/R-induced damage of HBMEC. (A) p66\textsuperscript{Shc} activation remains unchanged after exposure of HBMEC to hypoxia compared to normoxia (n=8). HIF-1α stabilization is used as an indicator for effective hypoxic condition. (B) Hypoxia followed by reoxygenation increases phosphorylation of p66\textsuperscript{Shc} at Ser36 compared to normoxia (n=6). (C) Representative immunoblot of p66\textsuperscript{Shc} silencing in vitro. Pre-incubation of HBMEC with p66\textsuperscript{Shc} siRNA selectively reduces p66\textsuperscript{Shc} levels, without affecting the levels of the two other Shc isoforms p52\textsuperscript{Shc} and p46\textsuperscript{Shc}. (D) H/R leads to an increased O$_2^-$ production (n=12-13) that is blunted after silencing of p66\textsuperscript{Shc} (n=7-13). (E) HBMEC exposed to H/R show a reduced NO bioavailability (n=12) that is elevated after p66\textsuperscript{Shc} silencing (n=6-12). (F–G) H/R does not alter phosphorylation of eNOS at both sites studied (Ser1177 and Thr495) and eNOS protein expression compared to normoxia (n=7). (H) Pre-incubation of HBMEC with p66\textsuperscript{Shc} siRNA reduces H/R-increased NADPH oxidase activity (n=11). Data are expressed as mean ± s.e.m. * P<0.05, ** P<0.01, *** P<0.001 for H/R vs. normoxia; # P<0.05, ## P<0.01 for H/R with sip66\textsuperscript{Shc} vs. H/R.

**Figure 5** Effect of p66\textsuperscript{Shc} silencing and ROS on H/R-decreased claudin-5 expression. (A) Immunoblot analysis reveals decreased claudin-5 protein levels after exposure of HBMEC to H/R (n=7), but not of occludin (n=5) (B) and VE-cadherin (n=7) (C). Pre-incubation with p66\textsuperscript{Shc} siRNA (n=7) (D), or apocynin (0.1 mmol/L) (n=6) (E), increases claudin-5 levels under H/R condition. Data are presented as mean ± s.e.m. ** P<0.01, *** P<0.001 for H/R vs. normoxia; # P<0.05, ## P<0.01 for H/R with sip66\textsuperscript{Shc} or apocynin vs. H/R.

**Figure 6** p66\textsuperscript{Shc} gene expression in PBM of ischaemic stroke patients. (A) Real-time PCR determined increased p66\textsuperscript{Shc} mRNA levels in stroke patients 6 h (n=27), but not 24 h (n=16) after initial stroke symptoms compared to the levels of control subjects (n=19). Data are expressed as mean ± s.e.m. (B) p66\textsuperscript{Shc} gene expression 6 h after initial stroke symptoms was determined in subgroups of stroke patients according as they
did (t-PA), or did not (w/o t-PA) receive thrombolytic treatment. Values from controls are also shown for comparisons. $p66^{Shc}$ mRNA levels are not different between both stroke subgroups (w/o t-PA: n=13; t-PA: n=14). Both subgroups show higher $p66^{Shc}$ levels compared to controls. Data are expressed as mean ± s.e.m. (C) Correlation between $p66^{Shc}$ gene expression and NIHSS at admission of all study subjects (n=46). (D–E) Correlation analysis of $p66^{Shc}$ transcripts and deltaNIHSS (NIHSS discharge – NIHSS admission) in w/o t-PA patients (n=13) and in t-PA patients (n=14). For (C–E) linear regression trend lines are illustrated. *** P<0.001. NIHSS, National Institutes of Health Stroke Scale; t-PA, tissue plasminogen activator.
Table 1 Characteristics of clinical study population. Clinical characteristics of controls and stroke patients do not differ statistically. Subgroup analysis shows statistically significant differences in age and NIHSS at discharge between patients treated with thrombolysis and patients which did not receive thrombolysis. Age is given as mean (with ranges) and NIHSS is expressed as mean ± SD. TIA, transient ischaemic attack; tPA, tissue plasminogen activator; TACI, total anterior circulation infarct; PACI, partial anterior circulation infarct; LACI, lacunar infarct; POCI, posterior circulation infarct; TOAST, Trial of ORG 10172 in Acute Stroke Treatment; NIHSS, National Institutes of Health Stroke Scale.