SUR1-TRPM4 channels, not K_{ATP}, mediate brain swelling following cerebral ischemia

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Abstract

Background.—Preclinical and emerging clinical data show that glibenclamide reduces space occupying edema and brain swelling following cerebral ischemia. Glibenclamide is a potent inhibitor of numerous sulfonylurea receptor (SUR)-regulated channels, including K_{ATP} (SUR1-KIR6.2, SUR2A-KIR6.2, SUR2B-KIR6.2, SUR2B-KIR6.1) and SUR1-TRPM4. Here, we used molecularly specific oligodeoxynucleotides (ODNs) to investigate the role of various SUR-regulated ion channel subunits in post-ischemic brain swelling.

Methods.—Focal cerebral ischemia was induced in adult male rats by permanent middle cerebral artery occlusion (pMCAo). We used this model to study the effects of antisense-ODNs (AS-ODNs) directed against *Abcc8*/SUR1, *Trpm4*/TRPM4, *Kcnj8*/KIR6.1 and *Kcnj11*/KIR6.2 on hemispheric swelling, with sense or scrambled ODNs used as controls. We used antibodybased Förster resonance energy transfer (immuno-FRET) and co-immunoprecipitation to study the co-assembly of SUR1-TRPM4 heteromers.

Results.—In the combined control groups administered sense or scrambled ODNs, pMCAo resulted in uniformly large infarct volumes (mean±SD: 57.4±8.8%; n=34) at 24 hours after onset of ischemia, with no effect of AS-ODNs on infarct size. In controls, hemispheric swelling was 23.9±4.1% (n=34), and swelling was linearly related to infarct volume (*P*<0.02). In the groups administered anti-*Abcc8*/SUR1 or anti-*Trpm4*/TRPM4 AS-ODN, hemispheric swelling was significantly less, 11.6±3.9% and 12.8±5.8% respectively (*P*<0.0001), and the relationship between infarct volume and swelling was reduced and not significant. AS-ODNs directed against

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Kcnj8/KIR6.1 and *Kcnj11*/KIR6.2 had no significant effect on hemispheric swelling (23.3±5.4% and 22.9±5.8% respectively). Post-ischemic tissues showed co-assembly of SUR1-TRPM4 heteromers.

Conclusions.—Post-ischemic hemispheric swelling can be decoupled from infarct volume. SUR1-TRPM4 channels, not K_{ATP}, mediate post-ischemic brain swelling.

Keywords

SUR1-TRPM4 channel; Sulfonylurea receptor 1 (SUR1); Transient receptor potential melastatin 4 (TRPM4); K(ATP) channel; cerebral ischemia; hemispheric swelling; cerebral edema

INTRODUCTION

Brain swelling due to cerebral edema is a widely recognized complication of cerebral ischemia. The harmful, often fatal effects of brain swelling are well known in patients with large hemispheric (malignant) infarction (LHI) [2, 5], but swelling also may complicate smaller strokes. Recent evidence indicates that swelling is an independent predictor of poor outcome in nonmalignant strokes, with a swelling volume of only 11 mL being the threshold with the greatest sensitivity and specificity for predicting poor outcome [4].

Glibenclamide was first shown to reduce cerebral edema (excess tissue water) in a rodent model of LHI [27]. Subsequent work with different models of LHI confirmed that glibenclamide reduces ischemic brain swelling and its associated high mortality [28–30]. In rodent models of both fatal (malignant) and non-fatal stroke, reducing edema by treatment with glibenclamide is accompanied by improved neurological scores [28–30, 38]. Data from two phase 2 clinical trials showed that intravenous glibenclamide reduces edema and brain swelling in patients with LHI [12, 13, 25, 26, 35].

Glibenclamide is a potent inhibitor of numerous ion channels that are regulated by sulfonylurea receptor (SUR), including four molecularly distinct K_{ATP} channels (SUR1-KIR6.2, SUR2A-KIR6.2, SUR2B-KIR6.2, SUR2B-KIR6.1) [24, 33, 34], as well as SUR1-TRPM4 channels [27, 40]. Here, we sought to determine which of these ion channels might be the target of glibenclamide in post-ischemic brain swelling. We employed a strategy of gene suppression of the various ion channel subunits using intravenous antisense oligodeoxynucleotides (AS-ODNs), which exhibit augmented efficacy at low pH [10], as found in ischemic brain [18]. We studied a permanent middle cerebral artery occlusion (pMCAo) model, in which swelling is robust but generally non-lethal [32, 37]. Here, we report that suppressing *Abcc8*/SUR1 and *Trmp4*/TRPM4, but not *Kcnj8*/KIR6.1 or *Kcnj11*/KIR6.2, was highly effective in reducing hemispheric swelling, consistent with involvement of SUR1-TRPM4, not K_{ATP}.

METHODS

Ethics statement.

We certify that all applicable institutional and governmental regulations concerning the ethical use of animals were followed during the course of this research. Animal experiments

were performed under a protocol approved by the Institutional Animal Care and Use Committee of the University of Maryland School of Medicine, and in accordance with the relevant guidelines and regulations as stipulated in the United States National Institutes of Health Guide for the Care and Use of Laboratory Animals. All efforts were made to minimize the number of animals used and their suffering.

pMCAo.

Male Wistar rats, aged 11–12 weeks (300–350 gm) (Envigo, Indianapolis, IN) underwent pMCAo using intra-arterial occluders (0.39 mm; catalogue #4039PK5Re; Doccol Corp, Redlands CA) and laser Doppler flowmetry to assure >75% reduction in relative cerebral blood flow, as we described [30, 31].

Treatments.

In three series of experiments, pMCAo rats were randomly assigned to receive either AS-ODN targeting a channel subunit, or scrambled ODN (Scr-ODN) or sense ODN (SE-ODN) as control, including *series 1*: AS-ODN targeting *Abcc8*/SUR1 (12 rats, with 13 Scr-ODN controls); *series 2*: *Trpm4*/TRPM4 (13 rats, with 16 SE-ODN controls); *series 3*: *Kcnj8*/KIR6.1 (7 rats) or *Kcnj11*/KIR6.2 (7 rats), with 5 Scr-ODN controls. Rat anti-*Abcc8*/SUR1 (5′-GGCCGAGTGGTTCTCGGT-3′), anti-*Trpm4*/TRPM4 (5'-TTGTGGTAACACTCTCCAAA-3') and anti-*Kcnj11*/KIR6.2 (5'-CCTTTCGGGACAGCATGGCT-3') AS-ODNs were described previously [9]. Rat anti-*Kcnj8*/KIR6.1 AS-ODN (5'-TCTTCCTGGCCAGCATCTTC-3'), which targets the translation initiation codon [7], was newly designed for the present study. ODNs, which were phosphorothioated at 4 distal bonds at both the 5' and 3' ends to protect against endogenous nucleases [8], were obtained commercially (Sigma-Aldrich). Prior to surgery, miniosmotic pumps (Alzet 2001D, 8 μL/hour; Durect Corp.) fitted with jugular vein catheters were loaded with ODN and primed. ODN solutions were prepared in sterile normal saline (NS) (4 mg/mL). Immediately after pMCAo, the jugular vein was catheterized, a loading dose of ODN (400 μg in 100 μL) was administered intravenously (IV), and the pump catheter was inserted into the jugular vein with the pump implanted subcutaneously in the dorsal thorax, to obtain IV delivery of ODN at a rate of 32 μg / hour until euthanasia, yielding a total dose delivered of ~1.2 mg / 24 hours.

Neurofunction.

At 24 hours, neurological function was evaluated by investigators blinded to treatment group using an 8-point neuroscore system (8, stroke-related death; 7, no response to stimulation; 6, ambulate only when stimulated; 5, circle or ambulate spontaneously only to the left; 4, spontaneous movements in all directions with circling to the left exhibited only if pulled by the tail; 3, reduced resistance to lateral push toward the left side; 2, left shoulder adduction when suspended by the tail; 1, left forelimb flexion when suspended by the tail, or failure to extend the right forepaw fully; 0, no neurological deficit), as we described [29]. Here, for analysis, scores were dichotomized at 5/6 based on the presence or absence of spontaneous movements. This threshold has high interobserver reliability, and was found previously to distinguish between treatment groups in the same pMCAo model [38].

Infarct volume and hemispheric swelling.

After evaluation of neurological function, rats were euthanized. Six coronal sections, 2 mm thick, were used to determine infarct volumes using 2,3,5-triphenyltetrazolium chloride (TTC), corrected for swelling, as described [16], and to determine hemispheric swelling, as described [16].

Exclusions.

A total of 86 rats underwent pMCAo. Of these, 3 were excluded prior to randomization for LDF <75%; 3 rats died after randomization, prior to planned euthanasia at 24 hours; 7 rats were excluded for TTC-infarct volumes <40%. These exclusions left 73 rats as the basis for this report, with group assignments detailed above (*see* "Treatments").

Immunohistochemistry, immuno-FRET and co-immunoprecipitation.

These procedures were performed using custom anti-SUR1 and anti-TRPM4 antibodies, as we described [9, 40].

Data analysis.

Data are presented as mean ± S.D., or as box plots (small box, mean; - -, median; large box, 25th and 75th percentile; ×, 1st and 99th percentile; -, max and min). Infarct volumes and hemispheric swelling were analyzed using ANOVA with post-hoc Bonferroni comparisons. The relationship between infarct volume and hemispheric swelling was analyzed using linear regression. Dichotomized neurobehavioral scores were analyzed using Fisher's exact test (2-tailed). Statistical analyses were performed using GraphPad Prism version 8. $P<0.05$ was considered to be statistically significant.

RESULTS

Infarct volume

Rats underwent pMCAo and were randomly assigned to receive AS-ODNs directed against *Abcc8*/SUR1, *Trpm4*/TRPM4, *Kcnj8*/KIR6.1 or *Kcnj11*/KIR6.2, with sense or scrambled ODNs used as controls. In the combined control groups, TTC infarct volumes at 24 hours were 57.4±8.8% (n=34) of the hemisphere (Fig. 1) [38], and were not different between individual control groups (P=1) (Fig. 2A). In the four groups treated with AS-ODNs, infarct volumes were unchanged compared to controls (P=1) (Fig. 1, 2A).

Hemispheric swelling

In the combined control groups, hemispheric swelling at 24 hours was 23.9±4.1% (n=34) [38], and was not different between individual control groups (P=1) (Fig. 2B). Of the four groups treated with AS-ODNs, compared to controls, hemispheric swelling was significantly decreased by anti-*Abcc8*/SUR1 ($P<0.0001$) and anti-*Trmp4*/TRPM4 AS-ODN ($P<0.0001$) (Fig. 1, 2B), but not by anti-*Kcnj8*/KIR6.1 (P=1) or anti-*Kcnj11*/KIR6.2 (P=1) AS-ODN (Fig. 2B).

In the combined control groups, linear regression showed that hemispheric swelling was significantly related to infarct volume (P<0.02) (Fig. 2C). By contrast, in rats administered anti-*Abcc8*/SUR1 and anti-*Trmp4*/TRPM4 AS-ODN, the regression line was shifted downward, and the slope was not significantly different from zero.

Neuroscores, dichotomized according to the presence or absence of spontaneous movements, reflected hemispheric swelling. Significantly greater proportions of rats exhibited spontaneous movements that had been administered anti-*Abcc8*/SUR1 or anti-*Trmp4*/TRPM4 AS-ODN, compared to Scr-ODN, SE-ODN, anti-*Kcnj8*/KIR6.1 AS-ODN, or anti-*Kcnj11*/KIR6.2 AS-ODN (Fig. 2D).

SUR1-TRPM4 heterodimers

Analysis of post-ischemic brain tissues showed *de novo* upregulation of SUR1 and TRPM4, which colocalized in microvessels, and which co-assembled into heteromers, as indicated by immuno-FRET (Fig. 3A–F). Co-immunoprecipitation confirmed the co-assembly of SUR1-TRPM4 heteromers, with quantification showing a >10-fold increase post-ischemia (Fig. 3G).

DISCUSSION

Glibenclamide is a potent inhibitor of four molecularly distinct K_{ATP} channels, SUR1-KIR6.2, SUR2A-KIR6.2, SUR2B-KIR6.2 and SUR2B-KIR6.1, some of which are expressed constitutively by various cell types in the brain [24, 33, 34]. Glibenclamide also is a potent inhibitor of SUR1-TRPM4 channels, which are not constitutively expressed, but are transcriptionally upregulated in all members of the neurovascular unit following cerebral ischemia [27, 39, 40]. In the case of these 5 ion channels, glibenclamide inhibits channel function by targeting the regulatory subunit, SUR. In addition, glibenclamide inhibits the NOD-like receptor pyrin domain containing 3 (NLRP3) inflammasome, an effect that is independent of K_{ATP} or SUR [43].

Historically, glibenclamide was considered to be a specific inhibitor of K_{ATP}, and so effects of glibenclamide post-ischemia, both favorable and potentially unfavorable, often were assumed to be due to blockade of K_{ATP}. Because K_{ATP} channels are reported to protect neurons from ischemia [33, 34], and to play an important role in ischemic preconditioning [3, 6], questions arose about potentially harmful effects of administering glibenclamide post-ischemia.

However, multiple reports have appeared demonstrating that post-ischemic administration of glibenclamide is beneficial. Glibenclamide inhibition of K_{ATP}, at least in theory, is said to reduce ATP consumption that would be required to replenish intracellular K^+ extruded via K_{ATP} in post-ischemic neurons [19]. Glibenclamide favorably influences microglial function, enhances neurogenesis, and improves neurological outcome post-ischemia, with these effects attributed to K_{ATP} blockade [20–22]. Glibenclamide ameliorates ischemia-reperfusion injury via modulating oxidative stress and inflammation [1]. In non-lethal rat models of stroke, glibenclamide reduces infarct volume [31, 38], in lethal rat models, glibenclamide reduces brain swelling and death [27–30, 44], and in both non-lethal and

lethal models, glibenclamide improves neurological outcome [28–30, 38, 42, 44]. Moreover, data from two phase 2 clinical trials show that intravenous glibenclamide reduces edema and brain swelling in patients with LHI [12, 13, 25, 26, 35].

In the last series of studies, the effects of glibenclamide were attributed not to K_{ATP}, but to SUR1-TRPM4. The initial work reporting involvement of SUR1-TRPM4 channels (at the time, named SUR1-regulated $NC_{Ca\text{-}ATP}$ channels) in cerebral ischemia used patch clamp techniques to show that post-ischemic neurons express ion channels with the biophysical properties of SUR1-TRPM4, not K_{ATP} [27]. Support for involvement of SUR1-TRPM4 came from subsequent work on post-mortem tissues from humans with stroke, in which immuno-FRET was used to demonstrate co-assembly of SUR1-TRPM4 heteromers in all cell types of the neurovascular unit [17]. Support for involvement of TRPM4 came from work with a murine model of stroke, in which global gene deletion of *Trpm4* yielded a 2-fold reduction in infarct volume and substantial recovery of motor function [15].

Here, we provide evidence that SUR1-TRPM4 is a critical target of glibenclamide in cerebral ischemia. The outcome measure that we studied – hemispheric swelling – was shown to be linked to SUR1-TRPM4, not K_{ATP}, based on experiments with molecularly specific AS-ODNs that targeted four channel subunits individually. Our experiments showed that hemispheric swelling was significantly reduced by AS-ODNs directed against *Abcc8*/SUR1 and *Trpm4*/TRPM4, but not *Kcnj8*/KIR6.1 or *Kcnj11*/KIR6.2, consistent with involvement of SUR1-TRPM4, not K_{ATP}, in post-ischemic brain swelling. Moreover, we showed here, as previously [17], that SUR1-TRPM4 heteromers co-assemble in post-ischemic tissues, consistent with the formation of functional SUR1-TRPM4 channels. The present results are arguably the strongest to date to distinguish between SUR1-TRPM4 and K_{ATP}, and to implicate SUR1-TRPM4 in post-ischemic cerebral edema.

The effect on hemispheric swelling of anti-*Abcc8*/SUR1 AS-ODN found here resembles closely the effect of glibenclamide in the same pMCAo model reported by an independent group, Wali et al. [38]. In both studies, control pMCAo rats showed 50–60% infarct volumes and 20–25% hemispheric swelling, and in both studies, swelling was reduced by approximately half, to 10–15% by treatments that targeted SUR1, either glibenclamide or anti-*Abcc8*/SUR1 AS-ODN. The major difference between the two studies was on infarct volume. Whereas the previous study by Wali et al. [38], as well as a report by us with the same model [31], showed significant reductions in infarct volumes with glibenclamide, here we found no effect of anti-*Abcc8*/SUR1 AS-ODN on infarct volume. Whether the lack of effect on infarct size with ODN reflects differential blood-brain barrier (BBB) penetration of ODN vs. drug, or some other difference, remains to be resolved.

It is generally accepted that infarct volume predicts hemispheric swelling [14, 31]. Most studies on treatments found to reduce post-ischemic edema are difficult to interpret regrading edema *per se*, since in most cases, the treatment also reduced infarct volume. By contrast, in the present study, the AS-ODNs that reduced hemispheric swelling had no effect on infarct volume, obviating this important confounder. In pMCAo rats administered control ODNs, we found a statistically significant positive correlation between infarct volume and hemispheric swelling, whereas in rats treated with AS-ODNs targeting *Abcc8*/SUR1 and

Trpm4/TRPM4, this relationship was lost, with the regression line shifted downward and the slope no longer significantly different from zero. To our knowledge, this is the first demonstration of frank decoupling of brain swelling from infarct volume.

Apart from hemispheric swelling [16], which we studied here, measures of edema reported in preclinical studies include excess tissue water, extravasation of endogenous or exogenous blood-borne molecules, tissue density by computed tomography, and relative diffusivity of water by magnetic resonance imaging. Excess tissue water has been criticized as potentially misleading, since small changes in percent water content actually reflect much larger changes in brain swelling [11]. Midline shift, which is used frequently in human studies [23], is used only rarely in preclinical studies, although it correlates well with excess tissue water [36]. Our preference for hemispheric swelling is based on the simplicity and reliability of this measurement, and its ability to convey the clinically relevant concepts of space-occupying edema and mass effect that give rise to neurological dysfunction.

This study has limitations. Although AS-ODNs offer a high degree of molecular specificity, they have limited penetration into the brain, owing to the negligible transport of these highly polar molecules across the BBB [41]. AS-ODNs are devoid of cellular specificity, and thus our data cannot address which element of the microvascular BBB complex – endothelium, pericyte, or astrocyte endfoot – is involved in SUR1-mediated post-ischemic brain swelling. Our experiments focused on brain swelling and its neurofunctional consequence, but did not examine microglial function, neurogenesis, oxidative stress, or inflammation, which also are reported to benefit from glibenclamide treatment post-ischemia. Thus, our conclusion on involvement of SUR1-TRPM4 in brain swelling cannot, without further study, be extended to these other domains.

In summary, the data presented here show that post-ischemic hemispheric swelling can be decoupled from infarct volume, and that SUR1-TRPM4 channels, not K_{ATP}, mediate post-ischemic brain swelling.

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Abbreviations:

AS-ODN	antisense oligodeoxynucleotide
ATP	adenosine triphosphate
BBB	blood-brain barrier
FRET	Förster resonance energy transfer
IV	intravenous
LHI	large hemispheric infarction

ODN	oligodeoxynucleotide
NLRP3	NOD-like receptor pyrin domain containing 3
NS	normal saline
pMCAo	permanent middle cerebral artery occlusion
Scr-ODN	scrambled oligodeoxynucleotide
SE-ODN	sense oligodeoxynucleotide
SUR	sulfonylurea receptor
TRP	transient receptor potential
TTC	2,3,5-triphenyltetrazolium chloride

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Highlights

- Glibenclamide reduces space occupying edema and brain swelling following cerebral ischemia.

- Glibenclamide inhibits a variety of SUR-regulated channels, including four distinct K_{ATP} channels and SUR1-TRPM4 channels.

- We used antisense oligodeoxynucleotides to investigate the role of K_{ATP} vs. SUR1-TRPM4 in post-ischemic brain swelling.

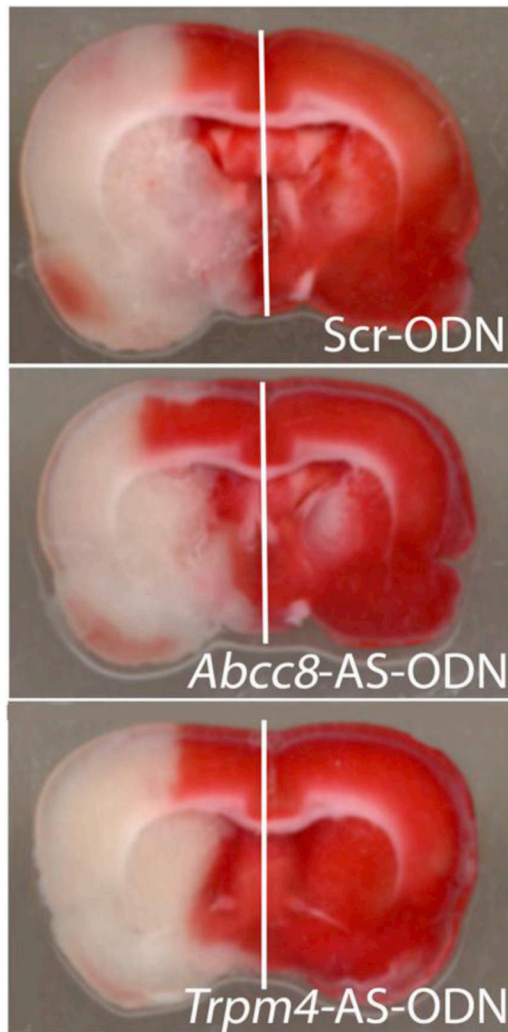- We found that SUR1-TRPM4 channels, not K_{ATP}, mediate post-ischemic brain swelling.

Figure 1. Coronal sections from the pMCAo model.
TTC-stained coronal sections show equivalently large infarcts, but reduced hemispheric swelling in rats administered AS-ODN targeting *Abcc8*/SUR1 and *Trpm4*/TRPM4, compared to control Scr-ODN.
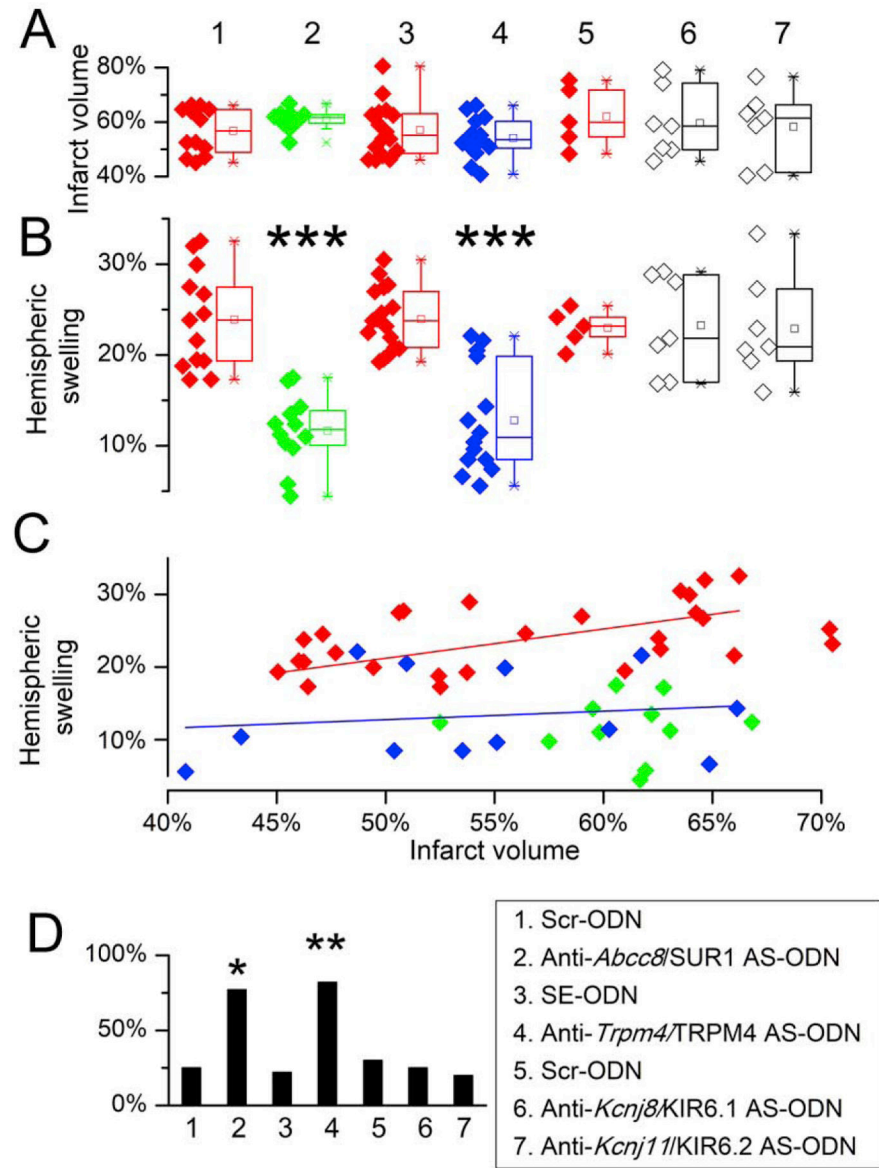
Figure 2. In the pMCAo model, post-ischemic hemispheric swelling is reduced by AS-ODN targeting *Abcc8*/SUR1 and *Trpm4*/TRPM4.
A: Infarct volumes at 24 hours in groups 1–7. **B:** Hemispheric swelling at 24 hours in groups 1–7; ***, $P < 0.0001$. **C:** Plot of hemispheric swelling vs. infarct volume for pMCAo rats administered control Scr-ODN or SE-ODN (red diamonds) or anti-*Abcc8*/SUR1 AS-ODN (green diamonds) or anti-*Trpm4*/TRPM4 AS-ODN (blue diamonds); linear regression for CTR data indicated a slope that was significantly different from zero ($P < 0.02$); linear regression for AS-ODN data indicated a slope that was shifted downward and was not significantly different from zero ($P = 0.35$). **D:** Percent or rats in each group that exhibited spontaneous (non-coerced) movements at 24 hours after pMCAo in groups 1–7; *, $P < 0.05$ for group 2 vs. 1; **, $P < 0.01$ and for group 4 vs. 3.
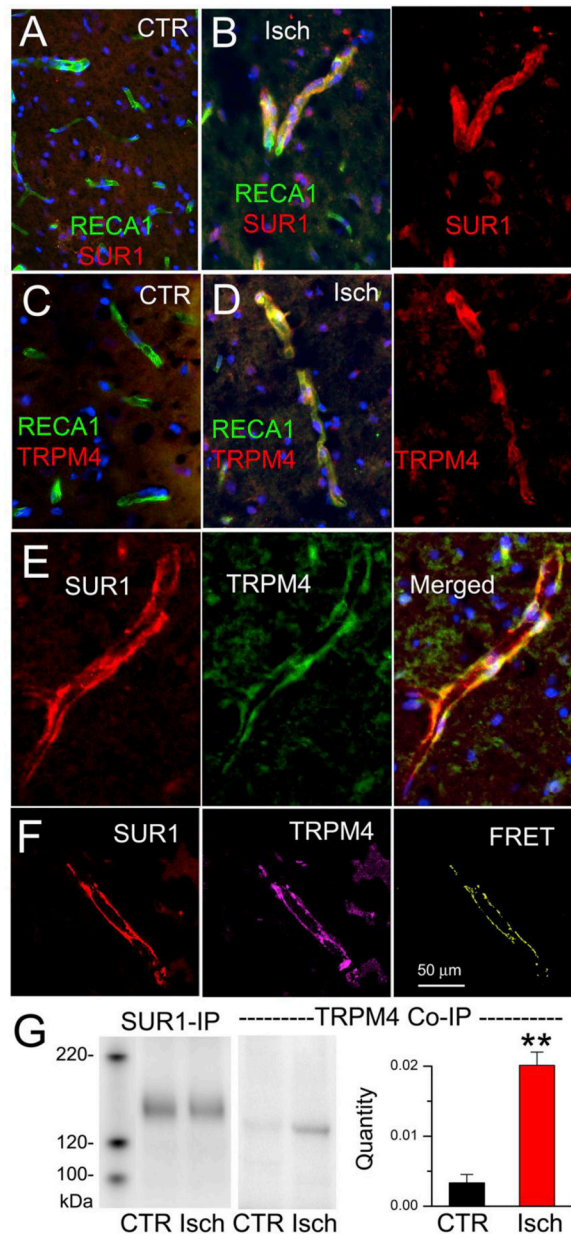
Figure 3. SUR1-TRPM4 is upregulated in microvessels post-ischemia.
A–D: Double immunolabeling of contralateral control (CTR) (A,C) and ipsilateral post-ischemic (Isch) (B,D) tissues for the endothelial marker, RECA1 (green), and SUR1 (red) (A,B), or RECA1 and TRPM4 (red) (C,D); superimposed images are shown in A; B, left panel; C; D, left panel. **E:** Double immunolabeling of ipsilateral post-ischemic tissues for SUR1 (red) and TRPM4 (green); superimposed images are shown at right. **F:** Double immunolabeling of ipsilateral post-ischemic tissues for SUR1 (red) and TRPM4 (magenta); FRET signal shown at right. **G:** Contralateral control (CTR) and ipsilateral post-ischemic (Isch) tissues underwent immunoisolation using anti-SUR1 antibody; the resultant isolate was immunoblotted using anti-SUR1 antibody (SUR1-IP) or using anti-TRPM4 antibody

(TRPM4 Co-IP); *bar graph:* quantity of SUR1-TRPM4 Co-IP normalized to HSC70; 3 replicates; **, P<0.01.