Sulfonylurea Receptor 1 in Humans with Post-Traumatic Brain Contusions

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Abstract

Post-traumatic brain contusions (PTBCs) are traditionally considered primary injuries and can increase in size, generate perilesional edema, cause mass effect, induce neurological deterioration, and cause death. Most patients experience a progressive increase in pericontusional edema, and nearly half, an increase in the hemorrhagic component itself. The underlying molecular pathophysiology of contusion-induced brain edema and hemorrhagic progression remains poorly understood. The aim of this study was to investigate sulfonylurea 1/transient receptor potential melastatin 4 (SUR1-TRPM4) ion channel SUR1 expression in various cell types (neurons, astrocytes, endothelial cells, microglia, macrophages, and neutrophils) of human brain contusions and whether SUR1 up-regulation was related to time postinjury. Double immunolabeling of SUR1 and cell-type–specific proteins was performed in 26 specimens from traumatic brain injury patients whose lesions were surgically evacuated. Three samples from limited brain resections performed for accessing extra-axial skull-base tumors or intraventricular lesions were controls. We found SUR1 was significantly overexpressed in all cell types and was especially prominent in neurons and endothelial cells (ECs). The temporal pattern depended on cell type: 1) In neurons, SUR1 increased within 48 h of injury and stabilized thereafter; 2) in ECs, there was no trend; 3) in glial cells and microglia/macrophages, a moderate increase was observed over time; and 4) in neutrophils, it decreased with time. Our results suggest that up-regulation of SUR1 in humans point to this channel as one of the important molecular players in the pathophysiology of PTBCs. Our findings reveal opportunities to act therapeutically on the mechanisms of growth of traumatic contusions and therefore reduce the number of patients with neurological deterioration and poor neurological outcomes.

Key words: brain contusion; brain edema; human; immunofluorescence; sulfonylurea receptor 1

Introduction

POST-TRAUMATIC BRAIN CONTUSIONS (PTBCs) are one of the most frequent lesions in patients with moderate or severe traumatic brain injury (TBI). PTBCs are traditionally considered primary injuries, but they have an inherent capacity to increase in size, generate perilesional edema, cause mass effect, induce neurological deterioration, and, in some patients, cause death. PTBCs are very dynamic lesions with a pathophysiology that is still challenging, and there are significant controversies in their clinical management. Contusions are frequently associated with volumetric expansion during the first 48 h post-trauma.^{1,2} In most patients, there is a progressive increase in pericontusional edema (edema progression), and in nearly half of patients, there is an increase in the hemorrhagic component itself (secondary hemorrhage or hemorrhagic progression).^{1,2}

Until recently, the underlying molecular pathophysiology of contusion-induced brain edema and hemorrhagic progression was poorly understood. However, new evidence has accumulated in the last decade that has shed light on the complex cellular and molecular mechanisms underlying volumetric increases resulting from edema and secondary hemorrhage in PTBCs. Simard and colleagues identified the crucial role of the sulfonylurea 1 (SUR1)/transient receptor potential melastatin 4 (TRPM4) ion channel in formation of brain edema in experimental models of ischemic lesions, spinal cord injuries (SCIs), and PTBCs.^{3–5} In addition, the landmark articles from Katayama and colleagues showed that the increased osmolarity in the core of contusions is a powerful attractor of water from pericontusional tissues. In pericontusional tissues, changes in the permeability of the blood–brain barrier (BBB) occur, which allow for rapid passage of ions and water into the contusion core.^{6,7}

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The traditional mechanism put forward for secondary hemorrhage (hemorrhagic progression) in PTBCs has been the existence of an overt or latent coagulopathy in patients with moderate and severe TBI. An alternative view proposed recently by Kurland and colleagues is that mechanical injury induces alterations in the microcirculation induced by the kinetic energy delivered to the brain tissue at the time of injury.⁸ In pericontusional endothelial cells (ECs), molecular mechanisms are set in motion that result in a significant increase in the permeability of the BBB to ions and water and, in some cases, result in its structural disruption with the passage of macromolecules and blood cells into the brain interstitium.^{8,9} Among these molecular mechanisms, up-regulation of SUR1-TRPM4 channels in the injured brain is emerging as one of great importance.^{3,5,8,10} In animal models, it has been shown that SUR1 is up-regulated in all cellular components of the neurovascular unit, mainly in ECs of the pericontusional brain.⁴ Blockade of SUR1 by glibenclamide or by antisense nucleotides significantly reduced progression of brain contusions in treated animals, compared to those treated with vehicle.^{4,11}

SUR1 is a protein of the family of adenosine triphosphate (ATP) binding cassette (ABC) transporters encoded by *Abcc* genes. Most ABC proteins couple ATP hydrolysis with translocation of molecules across biological membranes. SUR proteins are not transporters and they perform no recognized function by themselves.¹² To carry out its function, SUR requires an association with pore-forming subunits. The most studied association for SUR1 is with the pore-forming subunit, Kir6.2, to form the ATP-sensitive potassium (K_{ATP}) channel, which was discovered initially in cardiac myocytes¹³ and subsequently in other tissues, such as pancreatic β -cells, skeletal and smooth muscle, the brain, and the pituitary.¹⁴ SUR1 also associates with an ATP- and calcium-sensitive nonselective cationic channel recently identified as TRPM4 to form SUR1-TRPM4 channels (previously named SUR1-regulated $NCa-ATP$ channels).¹⁵ SUR1-TRPM4 channels transport all inorganic monovalent cations (Na^+ , K^+ , Cs^+ , Li^+ , and Rb^+), but are impermeable to Ca^{2+} and Mg^{2+} .¹⁶

SUR1 is constitutively expressed in some, but not in all, neurons of the central nervous system (CNS), where it forms K_{ATP} channels exclusively. However, SUR1 is constitutively absent in oligodendrocytes, astrocytes, and endothelium.^{17,18} SUR1 is transcriptionally overexpressed in neurons, astrocytes, oligodendrocytes, and ECs after different types of CNS injuries, including cerebral ischemia, TBI, SCI, subarachnoid hemorrhage (SAH), and in experimental models of germinal matrix hemorrhage.¹⁹ Expression of SUR1 has been investigated in many experimental models,^{4,10,16,19–23} but only four studies have been published for humans thus far, all of which were conducted with postmortem specimens and none with PTBCs.^{24–26}

Our aim in this study was to investigate the expression of SUR1 in human contusion specimens obtained at surgery from a cohort of TBI patients whose lesions were surgically evacuated. The main objectives were to investigate the expression of SUR1 in different cell types (neurons, astrocytes, ECs, microglia, macrophages, and neutrophils) and determine whether the abundance of SUR1 is related to the time that elapsed between the injury and the time of surgery. In this report, we present evidence that SUR1 is up-regulated in PTBCs in all brain cell types, consistent with the hypothesis that volumetric increases in PTBCs—both edema and hemorrhagic progression—are facilitated by SUR1 overexpression.

Methods

Clinical material and methods

This prospective study included all TBI patients who had an initial computed tomography scan and underwent surgical evacu-

ation of their brain contusions at our institution between January 2006 and July 2013. Brain specimens were obtained from surgically resected areas of the contusions and stored in a biobank collection of TBI samples at our institution (registration no.: C0002524 at the Carlos III Institute). Mean contusion volume at the time of evacuation was determined using the ABC/2 method.²⁷ As suggested by Iaccarino and colleagues, in patients with more than one lesion, the volume of each contusion was calculated and then added to get a total contusional volume.¹ Using the Extended Glasgow Outcome Scale, clinical outcome for each patient was assessed 6 months postinjury by an independent neuropsychologist (A.R.) blinded to the immunolabeling data.

The control group included tissue samples obtained from limited brain resections performed to access extra-axial skull base tumors or intraventricular lesions. These samples were included if the magnetic resonance imaging (MRI) scans did not show any abnormalities in T1-weighted, T2-weighted, or fluid attenuated inversion recovery images. Our research was carried out in accord with the Declaration of Helsinki of the World Medical Association.²⁸ This study and the tissue collection protocol were approved by the institutional ethics committee of Vall d'Hebron University Hospital (Barcelona, Spain; protocols PR-ATR-68/2007 and PR-ATR-286/2013), and written informed consent was obtained from all of the patients (including the controls) or the patient's next of kin.

Criteria for surgical evacuation of brain contusions

Surgical indications for evacuation of PTBCs are still controversial, and no evidence-based guidelines are available as yet. The only published guidelines regarding PTBCs, published by the Brain Trauma Foundation in 2006, include other focal or diffuse traumatic parenchymal lesions and thus are too general.²⁹ At our institution, patients must fulfill at least one of the following criteria to be considered for surgical evacuation: 1) For patients with intracranial pressure (ICP) monitoring, the ICP exceeds 20 mm Hg, and the total volume of a single contusion or multiple contusions exceeds the 25-mL threshold; 2) for patients without ICP monitoring (mostly moderate TBI), the total contusion volume (hemorrhagic and edematous components) is above the 25-mL threshold; 3) any temporal contusion that produces a significant mass effect and/or compresses the basal cisterns; and 4) contusions that induce a significant mid-line shift (greater than 5 mm) despite having an ICP <20 mm Hg. The surgical approach is usually excision of necrotic tissue without external bone decompression.

Brain tissue collection

After surgical resection, all specimens were transported to the laboratory on ice. Cauterized tissue and excess blood were eliminated by dissection in phosphate buffer. Specimens of a minimum size of $0.5 \times 0.5 \times 0.5$ mm were fixed with 4% paraformaldehyde for 48 h. Specimens were selected from areas of resected brain with preserved anatomical structure, corresponding to penumbral zones or the interface of penumbra/core using the terminology described by Kurland and colleagues.⁸ Tissues were cryoprotected using 30% sucrose and embedded in Tissue-Tek optimal cutting temperature compound (4583; Sakura Finetek Europe B.V., Alphen aan den Rijn, The Netherlands). From these blocks, 10- μ m sections were obtained using a cryostat (Leica CM3050 S; Leica Biosystems, Heidelberg, Germany), mounted on glass slides, and stored at -20°C until further analysis.

Controls

The control group included an initial cohort of 8 patients. MRIs of controls were independently assessed by two of the authors (J.S. and J.M.S.) blinded to immunolabeling data. Tissue quality was evaluated in hematoxylin-eosin staining sections by a

neuropathologist (E.M.S.) blinded to immunolabeling data using one scale for edema and another for signs of tissue hypoxia/ischemia (0: absent; 1: mild; 2: moderate; and 3: severe). Patients with edema and/or ischemia scores greater than 2 were excluded as controls. An important point to remark on is that because our controls were from patients surgically treated for extra-axial skull base tumors or intraventricular lesions, they should not be considered comparable to the controls used in animal models or those extracted from fresh human cadavers. We think it could be more appropriate to treat them equivalent to “sham” animals in experimental models. In addition, the extraction process, even under meticulous microsurgery, adds some degree of unpredictable damage and minimal inflammatory reaction to brain tissue.

Immunohistochemistry

Before immunolabeling, a pretreatment to reduce autofluorescence was performed by immersing the cryosections for 7 min in 0.1% sodium borohydride (NaBH₄) in 0.1 M of phosphate-buffered saline (PBS).^{30,31} Sections were incubated in a blocking solution containing 2% donkey serum (D9663; Sigma-Aldrich, St. Louis, MO) and 0.1% Triton-X (T8787; Sigma-Aldrich) in PBS 0.1 M for 1 h. Next, cryosections were incubated for 1 h at room temperature and then for 48 h at 4°C with the following primary antibodies: rabbit/goat anti-SUR1 (1:500; custom anti-SUR1 antibodies described by Woo and colleagues¹⁵), mouse anti-NeuN (1:200; MAB377; Millipore Corporation, Billerica, MA), mouse anti-glial fibrillary acidic protein (GFAP; 1:1000; CY3 conjugated; C-9205; Sigma-Aldrich), mouse anti-CD31 (1:100; M082329; Dako, Carpinteria, CA), mouse anti-CD68 (1:100; M081401; Dako), or rabbit anti-myeloperoxidase (MPO; 1:200; A039829; Dako). Fluorescent-labeled, species-appropriate secondary antibodies (Invitrogen, Eugene, OR) were used for visualization. Omission of primary antibodies served as a negative control. Sections were cover-slipped with polar mounting medium containing antifade reagent and the nuclear dye, 4',6-diamino-2-phenylindole (DAPI) (P36935; Invitrogen). Fluorescent signals were visualized using an epifluorescence microscope (FX100 Olympus or BX61 Olympus; Olympus Corporation, Tokyo, Japan), depending on analysis requirements.

Analysis of immunohistochemical findings

Quantitative immunohistochemistry: neurons and endothelial cells. To quantify SUR1-positive neurons and vessels, depending on section size, between 4 and 9 fields were captured for each specimen by using FSX-BSW software with an epifluorescence microscope (FX100 Olympus). For neurons, 600×450 μm² images were randomly captured from the cortex area of the sample. For vessels, 1200×900 μm² images were randomly captured from the whole sample. All of the obtained images were exported to ImageJ 1.47v (Wayne Rasband, National Institutes of Health, Bethesda, MD). Neurons (NeuN-positive cells) and SUR1-positive neurons (NeuN- and SUR1-positive cells) in each image were counted using the Image Cell Counter plugin (Kurt De Vos; <http://rsb.info.nih.gov/ij/plugins/cell-counter.html>), and the percentage of SUR1-positive neurons was calculated. For ECs, all of the CD31-positive structures and SUR1-positive vessels (CD31 and SUR1 positive structures) in each image were counted using the same plugin, and the percentage of SUR1-positive ECs was calculated. Percentages of SUR1-positive neurons and blood vessels in the different captured images for each specimen were calculated, and all of the analyzed fields were included in the final statistical analysis for each case.

Semiquantitative immunohistochemistry. Semiquantitative analyses were performed for GFAP, CD68, and MPO immunolabeling results. For these measurements, all of the sections were immunolabeled as a single batch for each cell-type-specific

marker and observed using an epifluorescence microscope. For this analysis, the presence or the amount of the different cell-type-specific markers was quantified in the whole section by the same observer (T.M.V.) using the following scale: −: absent; +: scanty; ++: moderate; and +++: numerous. Specific SUR1 immunoreactivity also was evaluated using the similar scale: −: none; +: in a few cells; ++: in many cells; and +++: in all or almost all cells. Median interobserver agreement of these scales for two independent observers was 0.83, with a minimum of 0.71 and a maximum of 0.88 depending on the cell analyzed.

Statistical analysis

Descriptive statistics were obtained for each variable. For continuous variables, the mean, median, range, and standard deviation were used for normally distributed data, and the median, minimum, and maximum values were used for non-Gaussian distributions. The Shapiro-Wilk's test and the inverse probability plot were used to test whether the data followed a normal distribution. Percentages and sample sizes were used to summarize categorical variables. To correlate two continuous variables, the Pearson's correlation test was used for data following a normal distribution, and the more conservative Spearman's rho was used if the data did not follow a normal distribution. For each marker, scatter plots were constructed with time as the abscissa and the percentage/score of positive immunolabeling as the ordinate. A simple linear regression and the ordinary least squares method were used. Adjusted *R*² values were calculated for all of the models to test whether linear or nonlinear models adequately explained the relationships between both variables. Statistical analyses were performed using R software (version 3.0.2)³² and the integrated development environment R Studio (v0.98.945).³³ The car package was used for regression analysis.³⁴ To calculate interobserver agreement between two independent observers using the immunolabeling ordinal scales, we used weighted kappa with the routine implemented in MedCalc (version 12.2; MedCalc Software, Mariakerke, Belgium). Statistical significance was considered when *p* ≤ 0.05. Data are presented graphically using box-and-whisker plots.

Results

Control group

Of the 8 initial controls, 5 were excluded because of pathological evidence of edema and/or ischemia that could have been caused by manipulation of tissues during surgery, suboptimal conditions during transport of samples to the laboratory, or pathology not detectable by MRI. Demographics of the 3 controls are summarized in Table 1.

Descriptive data of patients with contusions

A total of 26 contusion specimens were obtained from a cohort of 25 TBI patients (21 males and 4 females) ages 14–75 years

TABLE 1. DESCRIPTIVE DATA FOR CONTROL PATIENTS

Case	Age	Sex	Primary pathology	Tissue quality	
				Edema	Ischemia
1	57	F	Sphenopetroclival meningioma	0	1
2	32	F	Facial schwannoma	0	1
3	2	M	Rhabdoid tumor	1	2

Tissue quality scores (edema and ischemia): 0: absent; 1: mild; 2: moderate; and 3: severe.

F, female; M, male.

FIG. 1. Sulfonyleurea receptor 1 (SUR1) was overexpressed in neurons and capillary endothelial cells in pericontusional tissue. (A and C) Fluorescent double labeling for NeuN/CD31 (green) and SUR1 (red); merged images (NeuN/SUR1 or CD31/SUR1) in the right column in pericontusional tissue resected 36 h after trauma. (B and D) Double labeling with anti-NeuN antibody (green; B) or anti-CD31 antibody (green; D) and anti-SUR1 (red); merged images (NeuN/SUR1 or CD31/SUR1) in the right column, corresponding to case 2 in Table 1. Original magnification (A) and (B)=60 $\times$; (C) and (D)=40 $\times$. Nuclei were counterstained with DAPI. DAPI, 4',6-diamino-2-phenylindole.

(median, 52). Tissue samples were obtained at various times postinjury (median, 27 h; range, 5–99). Median contusion volume was 54 mL (range, 15–105). On admission, 16 (64%) patients scored above 9 on the Glasgow Coma Score scale and were thus included in the mild/moderate TBI category. Median clinical outcome assessed 6 months postinjury was heterogeneous (median, 4; range, 1–8). The small sample size was underpowered for conducting a robust statistical analysis to study the possible relationship between contusion volume or neurological outcome of patients and SUR1 expression.

Sulfonyleurea receptor 1-positive cells in the contusion and control groups

Neurons. Expression of SUR1 was analyzed in cortex samples of all patients. Median percentage of SUR1-positive neurons in

the analyzed fields was 23.2% (range, 11.0–30.4%) in controls and 71% (range, 17–97%) in contusions (Mann-Whitney's test, $p < 0.001$; Figs. 1 and 2). When the percentage of SUR1-positive neurons was plotted against time postinjury, a nonlinear trend of SUR1 positivity as a function of time was found; there was an increase in the number of SUR1-positive neurons in the first 24 h postinjury and stabilization of expression thereafter (Fig. 2).

Endothelial cells. CD31-positive cells were analyzed both in the gray and white matter of contusion and control tissues. Median percentage of CD31-positive SUR1-positive ECs in analyzed fields was 12% (range, 0–38.1%) for controls and 43% (range, 9.1–100%) for contusions (Mann-Whitney's test, $p < 0.001$; Figs. 1 and 3). Expression of SUR1 in ECs was higher in the white matter than in gray matter (Mann-Whitney's test, $p < 0.001$; Table 2) in both

FIG. 2. (Left) Box plots of the percentage of sulfonyleurea receptor 1 (SUR1)-positive neurons in contusions and controls. (Right) Scatter plot of SUR1-positive neurons (all the counts in analyzed images) versus time (hours) between injury and surgery. An increase in SUR1-positive neurons in the first 48 h was found, followed by stabilization. The solid curved line is a nonparametric regression smoother produced by the LOWESS function in R.

FIG. 3. (Left) Box plots of the percentage of sulfonyleurea receptor 1 (SUR1) CD31-positive cells in contusions and controls. Median percentage of CD31-positive SUR1-positive endothelial cells in the analyzed fields was 12% for controls and 42% for contusions (Mann-Whitney's test, $p < 0.001$). (Right) Time versus percentage of positive endothelial cells did not show a significant trend.

TABLE 2. EXPRESSION OF SUR1 IN ENDOTHELIAL CELLS FROM GRAY AND WHITE MATTER

Sample type	White matter + gray matter	White matter	Gray matter
Control	15.8 (2.3–21.4)	17.9 (9.1–21.4)	12.5 (2.3–19.2)
Contusion	43.4 (9.1–100)	63.8 (12.1–100)	33.5 (9.1–93.8)

Results are shown as median (min–max) of the percentage of SUR1-positive vessels versus total vessels in the sample, white matter, or gray matter. SUR1, sulfonylurea receptor 1;

control and contusion samples. Plot of time versus percentage of positive ECLs did not show a significant trend (Fig. 3).

Glial cells. Expression of SUR1 was not detected in GFAP-positive cells in controls (Table 3). Of the contusion specimens, only 4 (15.4%) did not have SUR1-positive cells. Of the remaining 22 specimens, 9 had mild-to-moderate and 13 had strong positivity for SUR1 (Fig. 4). The plot of time versus percentage of SUR1-positive glial cells showed a moderate increase in the number of SUR1-positive cells with time (Spearman's $\rho = 0.54$; $p = 0.002$; Fig. 5).

Macrophages and activated microglial cells. Occasional CD68-positive cells were found in 2 of the 3 controls, but none were SUR1 positive (Table 3). All contusion specimens contained a variable number of activated microglial cells/macrophages. Tissues from 4 contusions (16%) showed mild increases in the number of CD68-positive cells, relative to controls, whereas in most of them (84%), there was a moderate/high increase in the number of cells with the phenotype of activated microglial cells or macrophages. Many CD68-positive cells were also SUR1-positive (Fig. 4). The number of CD68/SUR1-positive cells increased with time post-injury (Spearman's $\rho = 0.62$; $p < 0.001$).

Neutrophils. In 2 of the 3 controls, neutrophils were identified, and 1 showed moderate SUR1-positive MPO cells. All contusion samples contained a variable amount of neutrophils. Approximately half of the contusion specimens (12 of 26) contained a significant number of MPO-positive cells. We did not find a relationship between time postinjury and the number of neutrophils observed in the specimens. Most neutrophils were SUR1 positive (Fig. 4). The number of MPO/SUR1-positive cells decreased as time between injury and surgery increased (Spearman's $\rho = -0.49$; $p = 0.011$; Fig. 5). We observed that most circulating

neutrophils were SUR1 negative, whereas those found in brain parenchyma were SUR1 positive (Fig. 6).

Discussion

In experimental models of brain contusions, a consistent, well-known fact is that the necrotic area suffers secondary growth in the first 24 h postinjury.³⁵ However, whether this phenomenon represents the inevitable expansion of a delayed primary injury or potentially avoidable secondary brain damage is a matter of clinical interest because it opens the opportunity to act on the mechanisms of growth and therefore of neurological deterioration and poor neurological outcome. A useful, biologically plausible model of PTBCs differentiates between the core of the contusion—that part of the brain that suffers irreversible damage immediately on impact—and the pericontusional tissue (or traumatic penumbra), similar to what was described in the classical neuropathological description of traumatic contusions by Lindenberg and Freytag in 1957 and by Astrup and colleagues in ischemic stroke.^{36,37} Lindenberg and Freytag observed that necrosis in the core can be found in postmortem examinations in patients who survived at least 3 h postinjury.³⁷ One of the compelling reasons to use this model is that it allows neuroscientists a better understanding of the complex physiopathology of post-traumatic focal lesions. An increase in brain edema is related to alterations of the permeability of the BBB, both in the core, where the BBB is rapidly destroyed, and in the pericontusional brain, where alterations in BBB permeability take place at an early stage with formation of ionic edema. In later stages, the BBB is structurally damaged and vasogenic edema, the passage of macromolecules, and blood cells to the extracellular space, and, finally, hemorrhagic progression occur.^{8,38,39}

The contusional core is characterized by rapid pannecrosis, disintegration, and homogenization of brain tissue.^{37,40} This tissue homogenate creates a powerful osmotic sink that can attract water to the contusion's core.^{6,7,41} Katayama and colleagues showed, in both experimental and clinical studies, that the core of the contusion acts as a powerful magnet for water because of high osmolality, which can reach 350–400 mOsm/kg.^{6,40,41} The increase in osmolality occurs very early after injury, and its main etiopathogenic mechanism is the increase in colloid osmotic pressure from the release of intracellular proteins and the production and liberation of idiogenic osmoles. They also showed that changes in inorganic ion concentrations are not relevant enough to justify the increase in water volume.^{40,42} The high osmolality in the core, which behaves as a single huge extracellular space,⁴¹ and the increase in the BBB's permeability to water in the pericontusional tissue, sets the pathophysiological scenario for volumetric increase in the PTBC.

The tissue around the core is vulnerable brain that has low flow and low oxygen availability and has important metabolic derangements, but preserves its structural integrity.^{43,44} In the pericontusional tissue, some researchers differentiate between penumbra, parapenumbra, and normal brain with the goal of separating the molecular mechanism involved in each zone.^{4,8} Pericontusional tissue absorbs part of the kinetic energy delivered at the time of injury, activating mechanoreceptors and downstream molecular pathways. In addition, pericontusional tissue continuously receives strong inflammatory signals from the core. Activation of free radicals of nitrogen and oxygen, cytokines, hypoxia-inducible factor-1 α , cyclo-oxygenases, and matrix metalloproteinases (MMPs) are the most important mediators of the inflammatory-induced response activated in perilesional tissue.⁴⁵

TABLE 3. SEMIQUANTITATIVE IMMUNOHISTOCHEMISTRY ANALYSIS

Cell type	Amount		SUR1	
	Controls	Contusion	Control	Contusion
Glial cells	+	++	–	++
Activated microglia cells/macrophages	+	++	–	+
Neutrophils	+	++	–	++

Amount: median count for the number of cells found; scale: –, absent; +, scanty; ++, moderate; +++, numerous. SUR1: average number of the total cells observed showing SUR1 immunoreactivity; scale: –, none; +, in a few cells; ++, in many cells; +++, in all or almost all cells.

SUR1, sulfonylurea receptor 1.

FIG. 4. Sulfonylurea receptor 1 (SUR1) was upregulated in glial cells, activated microglia/macrophages, and neutrophils in pericontusional tissue. (A and B) Fluorescent double labeling for GFAP (green) and SUR1 (red). (A) SUR1-positive glial cells in pericontusional tissue resected 32 h post-trauma. (B) Merged image for SUR1-negative glial cells in a control, corresponding to case 1 in Table 1. (C and D) Fluorescent double labeling for CD68 (green) and SUR1 (red). (C) High expression of SUR1 receptor in activated microglia/macrophages in pericontusional tissue resected 32 h post-trauma. (D) Merged image shows no expression of SUR1 receptor in activated microglia/macrophages in a control, corresponding to case 2 in Table 1. (E and F). Fluorescent double labeling for MPO (green) and SUR1 (red). (E) SUR1-positive neutrophils in pericontusional tissue resected 26 h post-trauma. (F) Merged image for SUR1-negative neutrophils in a control, corresponding to case 3 in Table 1. Original magnification, 40 $\times$. Original magnification (A) and (B)=40 $\times$; (C) and (D)=60 $\times$; (E) and (F)=100 $\times$. Nuclei were counterstained with DAPI. GFAP, glial fibrillary acidic protein; MPO, myeloperoxidase; DAPI, 4',6-diamino-2-phenylindole.

The molecular mechanisms of volume increase in contusions

Both brain edema and hemorrhagic progression share a molecular substrate that involves either functional or structural dysfunction of the BBB and of the neurovascular unit. This is the first report evaluating SUR1 expression in humans with PTBCs. We

found that SUR1 was significantly up-regulated in all types of brain cells with especially prominent increases in neurons, glia, and ECs. Microglia/macrophages and neutrophils also expressed SUR1. The temporal pattern of SUR1 expression was different in specific cells. In neurons, a significant increase was found within 24 h postinjury, after which expression reached a plateau. However, because of the pathophysiological importance of this trend,

FIG. 5. Scatter plot of the time course of sulfonyleurea receptor 1 (SUR1) immunoreactivity in contused tissue. (Left) Plot of SUR1-positive glial cells versus time (hours) elapsed between injury and surgery. (Right) Scatter plot of SUR1-positive microglia cells/macrophages versus time (hours) elapsed between injury and surgery.

further studies with larger sample sizes are needed to replicate and confirm this temporal profile in neurons.

A significant increase in CD68-positive cells (activated microglia/macrophages) was found, and their overexpression correlated positively with time. In all contusion samples, we observed neutrophils (MPO-positive cells), meaning that the BBB was structurally disrupted and allowed the passage of blood cells to the extracellular space. Most neutrophils were SUR1 positive and the number of positive cells decreased as time between injury and surgery increased. We observed that most circulating neutrophils were SUR1 negative, whereas those found in brain parenchyma were SUR1 positive. Our preliminary findings are in agreement with the experimental model of brain contusion in rats developed by Simard and colleagues, in which it was shown that SUR1 was up-regulated in neurons and ECs of the penumbra.⁴ In this model of focal cortical contusion, it was shown that there was no expression of SUR1 immediately postinjury, but that expression was robust in ECs and neurons 24 h postinjury.⁴ These findings need further verification, but if confirmed, indicate that the strongest inflammatory reaction occurs very early postinjury and is not delayed, as has been traditionally thought. Trauma, specifically PTBCs, causes brain tissue and cell damage and triggers very powerful inflammatory reactions that are mediated by what are known as “alarmins,” a series of endogenous molecules that are a subfamily of the larger family of damage-associated molecular patterns that signal tissue and cell damage.⁴⁶

Although we cannot rule out that our small sample size, cohort characteristics, and most important, variable elapsed time from injury to surgery might have influenced these temporal profiles, these trends, if verified in further studies, can help elucidate the pathophysiology of PTBCs.

The SUR1-TRPM4 channel is initially inactive, but when activated by ATP depletion in brain cells, leads to cell depolarization,

cytotoxic edema, and oncotic cell death.^{4,16} Activation of the channel in ECs increases the permeability of the BBB to ions, thus facilitating the formation of ionic and vasogenic edema in the first stage and the evolution to hemorrhagic progression when ECs suffer oncotic death and the BBB is structurally damaged.⁴ Therefore, up-regulation of SUR1, both in experimental models of brain contusions and in our study, points to SUR1 as one of the major molecular players in the pathophysiology of PTBCs. Blocking SUR1 in experimental models of contusions by glibenclamide or by antisense nucleotides largely reduced hemorrhagic progression and the final contusion volume and improved neurobehavioral functions of treated animals.^{4,11}

Sulfonyleurea receptor 1 implication in the volume increase of contusions. Most research post-TBI has focused on excitotoxicity, oxygen free radicals, ischemic and nonischemic forms of tissue hypoxia, metabolic disturbances, and neuroinflammation. However, despite its obvious importance in TBI, the mechanical stress of brain tissues has been relatively neglected as the initiator of many molecular pathways, probably because of the lack of understanding of how mechanical forces, by themselves, activate molecular cascades. There is increasing evidence that alterations in cell shape, induced by mechanical forces, activate several signaling pathways in most cells.^{19,47–49} Mechanical absorption of kinetic energy by the endothelium of pericontusional tissue, and the hypoxic environment created in that tissue by reduced cerebral blood flow and microvascular dysfunction, triggers a set of molecular mechanisms that increase the permeability pore of the BBB in the traumatic penumbra, promoting ionic and water flux through it.⁴⁸ In the case of SUR1-TRPM4, it is likely that both hypoxia and mechanical stress cause up-regulation of this channel that predisposes to edema and hemorrhagic progression.¹⁹

An increase in the permeability pore facilitates brain edema (cytotoxic, ionic, and vasogenic) and, in Simard’s concept, progression to end-stage edema: hemorrhagic conversion. It has been shown that hemorrhagic progression recruits viable tissue from the penumbra into the necrotic core of the contusion, increasing the size of irreversibly damaged brain.

In brain ischemia, it has been shown that edema and hemorrhagic conversion share molecular mechanisms that, if understood, would allow interference of the natural evolution of PTBCs by blocking these molecular targets.²⁴ Hemorrhagic progression in PTBCs is related to microvascular dysfunction of the penumbra.⁸ SUR1 has been associated with both edema formation and catastrophic microvascular dysfunction, indicating that it has a crucial role in the pathophysiology of PTBCs and that it can be an important therapeutic target.^{4,11} It is also associated with K_{ATP} channels in

FIG. 6. Sulfonyleurea receptor 1 (SUR1)-negative circulating neutrophils. Fluorescent double labeling for MPO (green) and SUR1 (red) in a pericontusional tissue resected 26 h post-trauma, corresponding to the same case shown in Figure 4E. Original magnification, 40 $\times$. Nuclei were counterstained with DAPI. MPO, myeloperoxidase; DAPI, 4',6-diamino-2-phenylindole.

pancreatic β -cells that are considered ATP/adenosine monophosphate sensors, coupling glucose metabolism to electrical activity and insulin secretion.¹⁴ Sulfonylureas stimulate insulin secretion by closing these channels.⁵⁰ In addition, SUR1 is the regulatory subunit of the SUR1-TRMP4 channel. Its overexpression is regulated by specific mechanisms that are crucial in the pathophysiology of PTBCs: 1) mechanical stress of the tissue; 2) early and delayed hypoxic events that contusions induce, both in the core and perilesional tissue; and 3) the strong power of the hyperosmolar core to attract water from the perilesional brain through a BBB that has increased ionic and water permeability. Whereas the last mechanism is a passive one, both mechanical injury and contusion-induced hypoxia activate a powerful transcriptional program in the pericontusional tissue with selective transcription factors that bind to specific DNA-binding domains, up- or down-regulating specific genes.⁵¹ As shown in the pivotal study by Vertraeten and colleagues conducted in Jurkat T cells, sublethal mechanical stress that does not disrupt the plasma membrane promotes alterations in its physical properties, modifies cytoskeleton structure, increases intracellular Ca^{2+} content, and activates several transcription factors.⁴⁷

In previous studies, we showed that, in PTBCs, a significant early increase in plasma levels in MMPs, specifically gelatinases (MMP-2 and MMP-9) is observed.⁵² Both gelatinases specifically degrade type IV collagen, laminin, and fibronectin, which are the major components of the basal membrane of cerebral blood vessels, and they therefore damage the BBB. Disruption of the basal membrane is probably the end stage of many common pathways that irreversibly damage the BBB.⁵³ Gelatinase levels decreased during the first 24 h postinjury, suggesting a burst of early systemic inflammatory responses, induced by the traumatic event.^{52,54} Therefore, the case for an early inflammatory response in brain contusions seems quite consistent. A better understanding of the role of the SUR1-TRMP4 channel in this early inflammatory response, and why SUR1 is overexpressed in cells that are immunological effectors (activated microglia and neurophilis) and why it participates in the early inflammatory response, opens new lines of research with significant translational value.

Sulfonylurea receptor 1 as a therapeutic target in post-traumatic contusions

The therapeutic management of PTBCs has been very limited to date. Though surgical evacuation is performed when lesions are volumetrically relevant or cause a significant shift or increase in ICP, no drug has been shown effective in improving the natural evolution of these lesions. The fact that SUR1 is up-regulated in these lesions opens new opportunities to modulate the molecular cascades generated by these focal injuries. Experimental evidence in rats shows that blocking SUR1 with low-dose glibenclamide or an antisense oligodeoxy nucleotide against *Abcc8* reduces SUR1 expression and hemorrhagic progression of the contusions.^{4,11} In animal models of ischemic stroke, SAH, and SCO, blocking the SUR1-TRMP4 channels, also resulted in significant reduction of final lesion size and significant improvement in neurological outcome.⁴ Whether or not blocking the same channel in human PTBCs will translate to improved clinical results in TBI remains to be shown in randomized, clinical trials.

Limitations of the study

The main limitations of our study were small sample size, nonhomogeneous time in which brain specimens were studied, and specimens used as controls. Most contusions blossom during the

first 72 h postinjury, and thus surgery is conducted at this early stage. Because of this, late human contusion specimens are difficult to obtain. This fact limits the interpretation of the temporal profile we found in up-regulation of SUR1 in PTBCs. A last, but important, limitation is the difficulty in obtaining optimal control specimens. Although we thought the type of control patients we selected for this study were the best we could get, the histological sections of most specimens explanted in the operating room by experienced neurosurgeons showed hypoxia and edema. That was the case even in those rapidly resected and manipulated in a much-reduced time frame. Although these early findings are difficult to explain, we had to exclude most of the controls because different amounts of edema and/or hypoxia were found in histological preparations of controls. In our opinion, this shows the exquisite sensitivity of the brain to any mechanical manipulation, to the isolation of the pial circulation needed for specimen extraction, and to the hostile environment of the manipulation and transport to the laboratory. Which of these factors is the most important one requires further studies that are also relevant for researchers using organotypic slices or primary cultures derived from the mature brain.

In the selected controls, we found a moderate increase in SUR1, specifically in ECs. In the experimental model of mild-to-moderate TBI conducted by Patel and colleagues in Long-Evans rats, sham animals (receiving craniotomy without cortical impact injury) presented some expression of SUR1 in ECs.⁴⁸ In the same article, the ipsilateral hippocampus of the moderately injured rats without macroscopic injury presented time-dependent up-regulation of the SUR1 protein,⁴⁸ confirming that any intervention (including surgery and brain tissue resection) can induce some up-regulation of SUR1 in the mammalian brain. Despite these limitations, mentioned in the Discussion section, we believe our control specimens were adequate “sham controls” and were probably better than specimens from cadavers. Our specimens underwent manipulations similar to those that were conducted in TBI patients, and therefore the 12% expression may have also been found in TBI patients. Despite these limitations, the increase of SUR1 in contusions was very significant and well above the threshold of the control sham group. Moreover, in the human brain, Mehta and colleagues found some expression of SUR1 in certain capillaries, venules, and arterioles in “normal controls” (brain specimens obtained from 4 men and 2 women who died from non-neurological diseases).²⁴ Four of the 6 controls expressed SUR1 in some capillaries.²⁴

The apparently better-quality specimens obtained in previous studies from controls who died of non-neurological disease is probably a reflection of the inability of dead brain tissue to initiate any response to tissue manipulation. Despite these limitations, we believe that the response found in our controls, with mild-to-moderate amounts of positive SUR1-positive cells, is similar to that which will occur in the resected specimens of contusions that were explanted at surgery under similar conditions. Therefore, we believe these controls are more representative of the manipulated brain than brain tissue explanted after death. We think that the significant differences in the SUR1 overexpression found between controls and specimens in our study increases the robustness of our findings.

Future research

Our study has focused on the study of SUR1, a receptor that is associated with an increase in permeability of the BBB by increasing the transcellular crossing of ions through the specialized transport systems (SUR1-TRMP4 channel) or by disrupting tight junctions as a consequence of early osmotic swelling and late necrosis of ECs.

Current evidence supports that TBI, and especially focal lesions such as PTBCs, induces an early and strong sterile inflammatory response, which is one of the driving forces in the pathophysiology of focal lesions and in disruption of the BBB. MMPs are increased early postinjury, and they are specific mediators of basal membrane disruption and are among the late effectors of BBB disruption.⁵² According to our data, SUR1 is up-regulated in activated microglia and in neutrophils that cross the BBB and reach the brain. What the exact role of SUR1 is in the neuroinflammatory cascade and relationship with other molecular pathways requires further research. There is need for a deeper understanding of the molecular pathways involved in the early leakiness of the BBB in PTBCs not only through transcellular channels, but also through the paracellular pathways and therefore the tight junctions. Both transcellular and paracellular zones of leakiness need to be studied to make sense of the puzzle that is the pathophysiology of PTBCs. We need to clarify how and when the abnormalities in function are accompanied by abnormalities in structure of the different proteins of the tight junctions, including claudins, occludin, and junctional proteins. This is crucial to understanding which molecular players are the main ones and which are bystanders of the increase in permeability and late structural disruption of the BBB. This, in turn, will help us understand the pathophysiology and therapeutic opportunities that alter the natural evolution of one of the most frequent, but neglected, lesions after human TBI. The main challenge in these patients, many with mild and moderate TBI, is to reduce the secondary damage and thus the number of patients who “talk and die” as well as the neurological sequelae in survivors.

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Author Disclosure Statement

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