Platelet-mediated changes to neuronal glutamate receptor expression at sites of microthrombosis following experimental subarachnoid hemorrhage

Laboratory investigation

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Object. Glutamate is important in the pathogenesis of brain damage after cerebral ischemia and traumatic brain injury. Notably, brain extracellular and cerebrospinal fluid as well as blood glutamate concentrations increase after experimental and clinical trauma. While neurons are one potential source of glutamate, platelets also release glutamate as part of their recruitment and might mediate neuronal damage. This study investigates the hypothesis that platelet microthrombi release glutamate that mediates excitotoxic brain injury and neuron dysfunction after subarachnoid hemorrhage (SAH).

Methods. The authors used two models, primary neuronal cultures exposed to activated platelets, as well as a whole-animal SAH preparation. Propidium iodide was used to evaluate neuronal viability, and surface glutamate receptor staining was used to evaluate the phenotype of platelet-exposed neurons.

Results. The authors demonstrate that thrombin-activated platelet-rich plasma releases glutamate, at concentrations that can exceed 300 μM. When applied to neuronal cultures, this activated plasma is neurotoxic, and the toxicity is attenuated in part by glutamate receptor antagonists. The authors also demonstrate that exposure to thrombin-activated platelets induces marked downregulation of the surface glutamate receptor glutamate receptor 2, a marker of excitotoxicity exposure and a possible mechanism of neuronal dysfunction. Linear regression demonstrated that 7 days after SAH in rats there was a strong correlation between proximity to microthrombi and reduction of surface glutamate receptors.

Conclusions. The authors conclude that platelet-mediated microthrombosis contributes to neuronal glutamate receptor dysfunction and might mediate brain injury after SAH.

key Words • subarachnoid hemorrhage • platelet • microthrombi • glutamate receptor expression • traumatic brain injury • vascular disorders

Abbreviations used in this paper: BSA = bovine serum albumin; CNQX = 6-cyano-7-nitroquinoxaline-2,3-dione; DAB = 3,3′-diaminobenzidine; D-AP5 = D(-)-2-Amino-5-phosphonopentanoic acid; FAST = Fast Analytical Sensing Technology; GluR2 = glutamate receptor 2; LTP = long-term potentiation; MEA = microelectrode array; PBS = phosphate-buffered saline; SAH = subarachnoid hemorrhage; RFU = relative fluorescence unit; SEM = standard error of the mean; TA-PrP = thrombin-activated platelet-rich plasma.
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...ter repeated depolarizations of glutamate receptors at synapses, causing an enhanced long-lasting increase in signal transmission. Cellular factors contributing to LTP include transportation of glutamate receptors to the membrane, phosphorylation and enhancement of single-channel conductance, and nuclear transcription with subsequent protein synthesis, leading to insertion of new postsynaptic glutamate receptors. Disruption of any of these mechanisms, particularly a loss of surface glutamate receptors, might contribute to the cognitive impairment characteristic of SAH survivors.

A number of phenomena occur following SAH that may impact glutamate receptor physiology and therefore influence LTP induction and/or cognitive performance. These include angiographic vasospasm, delayed cerebral ischemia, cortical spreading depression, and development of microthrombi. Microthrombi accumulate in parenchymal vessels after SAH and are readily viewable by immunohistochemical staining for their components (for example, fibrinogen) and their autofluorescence. They are of interest due to their presence in autopsy studies in patients who have died following SAH, but their relationship to the development of secondary neurological deficits in these patients is unknown.

Notably, platelet aggregation is of critical importance in microthrombi formation. The mechanisms leading to platelet aggregation might impact glutamate receptor signaling, as platelets are thought to release glutamate as part of their cell-cell signaling and have been shown to escape into the neuronal parenchyma after SAH. Here, we hypothesized that microthrombi contribute to neuron dysfunction after SAH through local glutamate release during platelet aggregation. Through mechanisms of excitotoxicity or downregulation of glutamate receptors, we hypothesized that local, platelet-mediated glutamate release during microthrombus formation might impact neuronal signaling and survival, thereby contributing to secondary injury following SAH.

**Methods**

All procedures described herein were approved by the Animal Care Committee at St. Michael's Hospital and complied with regulations of the Canadian Council on Animal Care.

**Assay of Glutamate Release in Thrombin-Activated Platelet-Rich Plasma**

Thrombin was diluted in 0.1% bovine serum albumin (BSA, made with Millipore ultra-filtered water; pH 6.5) to a concentration of 0.5 U of thrombin per 50 μl. Aliquots of diluted thrombin were stored in plastic Eppendorf tubes at −20°C until defrosted on ice for use. Preliminary tests indicated that 1.5 μl of thrombin were required for thrombin-evoked glutamate release from platelets.

**Microelectrode Array Preparation.** Ceramic-based microelectrode arrays (MEAs) that contained 4 platinum recording surfaces (15 μm × 333 μm) in a paired configuration were prepared to measure glutamate. These electrodes were fabricated and selected for recordings in vitro using published methods. Platinum sites 1 and 2 were coated with a solution containing glutamate oxidase, BSA, and glutaraldehyde, enabling these sites to selectively detect glutamate levels with low limits of detection. Platinum sites 3 and 4 were coated with only BSA and glutaraldehyde and served as sentinels, recording everything channels 1 and 2 record except for glutamate. All 4 recording sites were electroplated with a size-exclusion layer, 1,3-phenylenediamine. In the presence of glutamate oxidase, glutamate is broken down into α-ketoglutarate and hydrogen peroxide (H₂O₂). The H₂O₂ traverses the 1,3-phenylenediamine layer and is readily oxidized and recorded as current using the FAST-16 instrument (Quanteon).

**Microelectrode Array Calibration.** The microelectrode tip was submerged in 40 ml of room temperature 0.05-M phosphate-buffered saline (PBS, pH 7.1–7.4) and was stirred using a magnetic stir bar and battery-operated stir plate. Following 20 minutes of equilibration, aliquots of stock solutions in the amount of 500 μl ascorbic acid (20 mM), three 40-μl aliquots of L-glutamate (200 mM), and 40 μl H₂O₂ (8.8 μM) were added to the PBS to calibrate the MEA to produce final concentrations of 250-μM ascorbic acid; 200-, 400-, and 600-μM glutamate; and 8.8-μM H₂O₂. From the calibration, the slope (electrode sensitivity to L-glutamate), selectivity (capabilities of recording glutamate over ascorbic acid), and limit of detection (smallest amount of detectable glutamate) were determined. The average value for slope was 3.5 ± 0.3 pA/μM, for selectivity was 50 ± 11 to 1, and for the limit of detection was 3.1 ± 2 μM (6 electrodes; 12 glutamate recording sites).

**Amperometry.** Amperometric recording procedures were similar to published methods. Constant-voltage amperometry was performed using a FAST-16 MKI electrochemistry instrument (Quanteon) using Fast Analytical Sensing Technology (FAST) software (Quanteon) developed for concurrent 4-channel recording. For in vitro recordings, a potential of +0.7 V was applied versus an Ag/AgCl reference electrode, and the data were recorded at a frequency of 2 Hz. Current signals were converted to voltage by the headstage (2 pA/mV) and a secondary gain of 10 times was then applied for a final gain of 0.2 pA/mV.

**Measurements of Thrombin-Evoked Glutamate In Vitro.** A small plastic “cap” was used for experiments. One milliliter of the 1:1 platelets and Tyrode’s solution were added to the cap. A small magnetic stir bar set at the lowest possible speed was used to ensure rapid diffusion of the thrombin. The MEA was lowered into the solution and allowed to stabilize for up to 10 minutes. Thrombin was added (1.5 U), and the glutamate response was recorded over time. Following the glutamate response to thrombin, exogenous glutamate was added to verify MEA responsiveness. Then the voltage was dropped to 0.2 V (unable to oxidize H₂O₂) to ensure the specificity of the MEA for glutamate.

**Isolation and Dissociation of Cortical Cell Cultures**

Cortical cultures containing both neurons and glia...
were prepared on 12-well plates from embryonic Day 17 Wistar rats as previously described. Briefly, cerebral cortices were dissected from whole brains using microdissection forceps. Cortical cells were isolated from a cell suspension and seeded in plating medium (neurobasal medium containing 2% B-27 supplement, 1% fetal bovine serum, 0.5 mM L-glutamine, 25 μM glutamic acid, Invitrogen) onto poly-L-lysine (5 μg/ml; Sigma-Aldrich)-coated plates at a density of 1 x 10^6 cells/well, confirmed via hemocytometer-mediated cell counts. Four days after isolation, cells were fed with fresh maintenance medium (neurobasal medium containing 2% B-27 supplement, 0.5-mM L-glutamine, Invitrogen) containing 10-μM mixture of uridine and (+)-5-fluor-2′-deoxyuridine (1:1 ratio, Invitrogen) to halt the growth of glial cells.

At Day 11–14, cells were incubated with 10 μg/ml propidium iodide to quantify baseline cell death. A Vector V multiwell plate scanner (PerkinElmer) controlled by Workout software (Dazdaq) recorded the relative fluorescence units, which were used as a quantitative measurement of cell death, with greater fluorescence values corresponding to greater propidium iodide reactivity and therefore cell death. All parameters were kept constant by using the same protocol for all groups (the scanning area remained constant, as did the duration). Cells were then incubated for 1 hour with thrombin-activated platelet-rich plasma (TA-PrP, with or without glutamate receptor antagonists), washed with buffer, and propidium iodide readings were taken 20 hours later. Cell death in each condition was compared with wells exposed to 1-mM glutamate for 1 hour, which produces nearly 100% cell death. The control cells in these experiments were incubated in the same vehicle media used for the generation of TA-PrP, unexposed to activated platelets.

GluR2 Immunostaining In Vitro

Cells were fixed in 4% paraformaldehyde for 10 minutes, washed with 3 times for 5 minutes each time, and blocked with 10% normal goat serum at room temperature for 1 hour. Primary monoclonal n-terminal mouse anti–rat glutamate receptor 2 (GluR2) antibody (1:100, Millipore) was added, and cells were incubated overnight at 4°C. Cells were washed with PBS and secondary antibody (goat anti–mouse antibody, Alexa Fluor 488-conjugated, 1:1000, Millipore) were diluted and applied with 3% normal goat serum. Cells were incubated at room temperature for 1 hour in the dark. To minimize nonspecific reactivity, cells were washed thoroughly again and mounted. The absence of cell permeabilization as well as ensuring the epitope was n-terminally located allowed us to visualize only surface receptors. Blinding, as well as random selection of neurons under brightfield optics, ensured the absence of biases during data analysis. Fluorescence images were captured using a confocal microscope (Nikon Eclipse E100 equipped with a Radiance 2100, laser scanning system, Bio-Rad) controlled by Lasersharp 2000 software (Carl Zeiss). All parameters for capturing in each channel were kept constant among images (capture speed, laser intensity, number of passes, and optical filtering).

SAH Model

Male Sprague-Dawley rats (250–350 g) were randomly assigned to either saline injection or SAH. The SAH model is described elsewhere. In brief, rats were anesthetized with ketamine/xylazine (100/10 mg/kg), and a bur hole was drilled 7.5 mm anterior to the bregma in the midline. The tail artery was catheterized with a 0.75-inch 24-gauge catheter (AngioCath, BD Biosciences), and SAH was induced by injecting 250 μl of fresh blood withdrawn from the catheter through a 27-gauge spinal needle into the prechiasmatic cistern. The injection duration was 17 seconds, and injection was controlled using a syringe pump (Harvard Apparatus). We have previously developed a method that ensures correct placement of the needle in the subarachnoid space by advancing the needle until it touches the skull base and then withdrawing it 0.5 mm. Fluid resuscitation was given, and the rats were placed in an incubator maintained at 26°C during recovery. Saline control animals underwent injection of 250 μl of 0.9% NaCl. Body temperature and blood pressure were maintained throughout all procedures with appropriate monitors.

GluR2 Immunostaining in Brain Slices

Slides were deparaffinized and rehydrated through xylene and degrading ethanol solutions. Antigen was first retrieved in 0.01-mmol/L sodium citrate (pH 6.0) at 96°C. We used 0.3% H2O2 in water for 45 minutes to quench endogenous peroxidase activity. Slides were placed in 10% horse serum and 1% BSA in PBS, and incubated with primary antibody (mouse anti–rat GluR2, 1:400 in 1% BSA/PBS, Millipore) for 90 minutes. After washing with PBS, they were incubated with biotinylated secondary antibody (horse anti–mouse antibody 1:200 in 1% BSA/PBS, Vector Laboratories) for 30 minutes. Staining was visualized with VIP using the VECTASTAIN 7 ABC Kit (Vector Laboratories) and counterstained with 0.5% methyl green. To quantify the density of GluR2 staining in relation to microthrombi, each cell was assigned a numeric value representing its distance from the center of the thrombosed vessel; this was then correlated with the intensity of cytosolic 3,3′-diaminobenzidine (DAB) reactivity that would represent GluR2. As the brain is a 3-dimensional network of microvessels, we obtained 20-μm coronal cross-sections of each vessel, measured the length of the cross-section, and assigned the mathematical center as the measurement point to each cell body. For analysis, only vessels oriented in the coronal plane (that is, traveling along the medial-lateral axis) were used.

Source of Chemicals

All chemicals were purchased from Sigma-Aldrich unless stated otherwise. D(-)-2-Amino-5-phosphonoenoic acid (D-AP5) and 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) were from Tocris.

Statistics

All data are expressed as mean ± standard error (SEM). For analysis between 2 groups, the Student t-test was used. One-way analysis of variance (ANOVA) fol-
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allowed by a Student-Newman-Keuls post hoc correction was applied to normally distributed data to assess group differences. A p value smaller than 0.05 was considered to be significant. Linear regression analysis was used to assess the correlation between microthrombi and surface GluR2 expression.

Results

Thrombin-Induced Glutamate Release From Platelet-Rich Plasma

We first determined whether local glutamate concentrations rise during thrombin-induced platelet aggregation using electrochemical detection of glutamate. Baseline glutamate levels in plasma diluted in Tyrode’s buffer (1:1) were 216.9 ± 48.5 μM. After addition of 1.5 U of thrombin, the maximum peak response of glutamate was 336.5 ± 73.0 μM; an average 120-μM increase in glutamate concentrations (paired 2-tailed t-test: t5 = 4.2; p = 0.009, n = 6, Fig. 1). The measuring conditions and volumes are similar to those used by other investigators and these concentrations are consistent with some measurements in vivo. This was demonstrable evidence that upon activation by thrombin, platelets release glutamate as part of their aggregation process.

Thrombin-Activated Plasma is Neurotoxic

Based on these results we sought to determine whether TA-PrP was neurotoxic, and if so, whether this effect was mediated by activation of glutamate receptors. We stimulated exogenous clot formation with thrombin ex vivo and applied the activated platelet supernatant fluid to neuronal cultures for 1 hour (in the presence and absence of glutamate antagonists D-AP5 and CNQX to antagonize AMPA and NMDA receptors, respectively). Cells were washed, and propidium iodide readings were taken at 20 hours. One-way ANOVA demonstrated significant differences between our treatments (F = 3.987, p = 0.013). At a concentration of 1:4 (platelet-rich plasma/extracellular fluid), TA-PrP induced a marked increase in propidium iodide fluorescence at 20 hours (38.6 ± 6.8 in relative fluorescence units [RFU] over control cells (Fig. 2, p < 0.05). At a lower concentration of 1:20, there was an equivalent level of cell death (41.7 ± 5.9 RFU, p < 0.05 vs control, p > 0.05 vs 1:4). In both scenarios, there was a decrease in cell death with coapplication of glutamate antagonists, although this was not statistically significant as determined by Student-Newman-Keuls post hoc testing (29.0 ± 13.5 RFU and 30.4 ± 10.5 RFU respectively, p > 0.05). The levels of cell death following application of TA-PrP were equivalent to that produced with 50-μM glutamate (34.2 ± 9.3 RFU, p > 0.05 vs both concentrations of TA-PrP, Fig. 2). We concluded from this assay that although TA-PrP was neurotoxic, this was likely due to the presence of factors in addition to glutamate in the activated plasma, such that glutamate receptor antagonism was insufficient for mitigating cell death entirely.

Activated Platelets Reduced Surface Glutamate Receptor Expression

We sought to investigate additional mechanisms of neuronal glutamate receptor dysfunction, based on our previous findings that LTP was markedly impaired following SAH in rats and was associated with cognitive changes during behavioral analyses. Glutamate not only induces neuron death when applied in excitotoxic concentrations, but also induces a marked synaptic depression due to internalization of surface glutamate receptors. We conjectured that this might be a contributing factor to the dysfunction of brain parenchyma surrounding microthrombi after SAH, and as a surrogate for this, we quantified surface GluR2 receptor staining, the predominant glutamate receptor subunit expressed on the neuronal surface. We divided neuronal GluR2 fluorescence intensity by neuronal area, to give an average value per cell. Here, we found that TA-PrP markedly reduced surface GluR2 staining (a 43% reduction in fluorescence compared with control), an effect that was significantly attenuated by glutamate receptor antagonism (13% reduction versus control, one-way ANOVA: F = 6.88, p =
0.004, Fig. 3). Post hoc testing (Student Newman-Keuls) revealed a significant difference between control cells and those treated with platelet-rich plasma (q = 5.106, p = 0.003), but in the presence of glutamate receptor antagonists this effect was completely attenuated (q = 1.5, p = 0.298). We concluded that activated platelets markedly reduce surface glutamate receptor expression, an effect likely downstream of excessive receptor stimulation.

Reduced Surface Glutamate Receptor Expression Colocalizes With Microthrombi

The finding that activated platelets release glutamate and that platelet-rich plasma downregulates GluR2 surface expression may be relevant to the pathogenesis of SAH, since glutamate receptors play an integral role in memory consolidation and executive functioning. We sought to investigate whether these findings are recapitulated in a rat model of SAH.13,21,23 We previously showed marked accumulation of microthrombi in cortical and hippocampal capillaries, demonstrated by fibrinogen immunostaining, in this model.13 These microthrombi are also auto-fluorescent and readily viewable via fluorescence microscopy. However, how they relate to learning deficits is unknown. Thus, we combined images of microthrombi with DAB immunostaining for surface GluR2 expression. At 6 days post-SAH, we observed an immediately recognizable trend toward increasing GluR2 receptor expression radiating outward from the sites of microthrombosis. That is, there was a clear linear relationship between distance from the microthrombus and surface GluR2 levels (Fig. 4, R² = 0.70, n = 51, p < 0.01). Neurons adjacent to the clotted vessels had marked reductions in surface GluR2. We repeated this analysis for the relationship between neurons and vessels that did not contain microthrombi, and did not observe any relationship between distance from the vessel and GluR2 expression (R² = 0.1, n = 32, p > 0.05). These findings suggest that platelet-mediated glutamate release at sites of microthrombosis leads to a loss of glutamate receptor expression.

Discussion

The relationship between microthrombi and clinical deficits following aneurysmal SAH is currently unknown. This study is among the first to describe a direct relationship between microthrombosis and changes to the molecular biology of nearby neurons, which might contribute to the cognitive impairments observed in patients with SAH, such as difficulties with learning and memory, confusion, and mood alterations.2 Here, we have shown in two models that activated platelets induce changes in the phenotype of surface glutamate receptors and that proximity of neurons to microthrombi is a mediator of glutamate receptor expression.
Our current hypothesis is that platelet aggregation during microthrombotic events exposes surrounding neurons to glutamate. This could occur in one of two ways: either there are changes in the permeability of the blood-brain barrier at sites of microthrombi (as glutamate ordinarily does not cross the blood-brain barrier) or the platelets themselves escape into the neuronal parenchyma and release glutamate during their lysis or aggregation. Indeed there is evidence for occurrence of the latter after SAH, initiated by platelet-mediated release of collagenase and subsequent depletion of collagen IV in vessel walls, with escape of platelets into the brain. However, it is also known that the blood-brain barrier breaks down after SAH, which might allow for exposure of neurons to intravascular glutamate at sites of microthrombi. There is another scenario that might also contribute to the finding that GluR2 expression decreases around microthrombotic vessels. Currently it is unknown what effect these microcirculatory changes have on tissue oxygenation after SAH. It is plausible that there is local ischemia of tissue perfused by the ordinarily patent vessels that become sites of microthrombosis. In this scenario, glutamate concentrations might rise (and therefore GluR2 surface expression might fall) from neuronal release rather than platelet release. It is well established that ischemia results in excitotoxicity from failure of membrane-bound Na+/K+ pumps, local depolarization, and uncontrolled release of glutamatergic vesicles. Moreover, we also know that exposure of neurons to even modest increases (20 μM) in glutamate induces a marked downregulation of surface glutamate receptors via endocytotic machinery. Thus, it is important to investigate whether there is adequate tissue perfusion at sites of microthrombi.

It must be noted that in this model there is relatively limited cell death. This suggests that overt neuron loss is unlikely to explain the observed clinical deficits seen in these animals. These data support the hypothesis that platelet-derived glutamate impairs expression of glutamate receptors.
mate receptors and their function rather than killing the neurons. This may be responsible for the clinical deficit. A similar situation occurs in other neurological disease such as anti-NMDA receptor encephalitis, where there is a reduction of surface glutamate receptors but preserved neuron survival.

Ultimately the question remains whether the loss of surface GluR2 protein translates to neuronal dysfunction or clinical deficit. It is essential to perform whole-cell patch clamp recording of neurons surrounding microthrombi to examine their basal AMPA receptor activity or their miniature excitatory postsynaptic current amplitude to see if their activity is indeed depressed near microthrombi. Further experiments are needed, including treatment of SAH animals with inhibitors of GluR2 endocytosis. We previously designed these compounds for use in a traumatic brain injury model and have found that they inhibit the trafficking of GluR2 protein both in vitro and in vivo. It will be potentially important to test whether preserving AMPA receptor expression translates to improvement on cognitive performance assays, which we have previously shown are impaired after experimental SAH.

**Fig. 4.** Subarachnoid hemorrhage in vivo reduces GluR2 staining adjacent to microthrombi. A: Neurons are from animals 6 days post-SAH. Representative images showing autofluorescent microthrombotic vessels (white circle), GluR2 DAB reactivity, and DAB masks that were generated in ImageJ to quantify integrated optical density (IOD) of GluR2 staining per cell. Note the marked reduction of staining near the thrombus, with recovery of signal as the neurons radiate outward. Scale bar = 50 µm. B: 40× images of the vessels shown in A. Two examples are shown of thrombotic vessels with both adjacent neurons (left panels, approximately 30 µm from the vessel) and neurons farther away (approximately 100 µm). There is a paucity of staining next to the vessels. C: No such relationship exists between neurons and nonthrombosed microvessels. There is abundant GluR2 staining both near and away from these vessels. Bars = 10 µm. D: Linear regression analysis demonstrated a marked relationship between surface GluR2 expression and distance from microthrombi (upper graph, $R^2 = 0.6985$). There is no correlation between distance from clean vessels and GluR2 expression (lower graph, $R^2 = 0.0997$).
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Conclusions

These experiments show that platelets release glutamate in vitro in response to application of thrombin. This is expected to occur in vivo during formation of microthrombi after SAH. In a rat model of SAH, we found evidence for glutamate-mediated effects around microthrombi in the brain. We suggest a novel mechanism in which platelet-mediated microthrombosis contributes to neuronal glutamate receptor dysfunction and brain injury after SAH.

Disclosure

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