ORIGINAL RESEARCH ARTICLE

Traumatic Brain Injury Impairs Systemic Vascular Function through Disruption of Inward-Rectifier Potassium Channels

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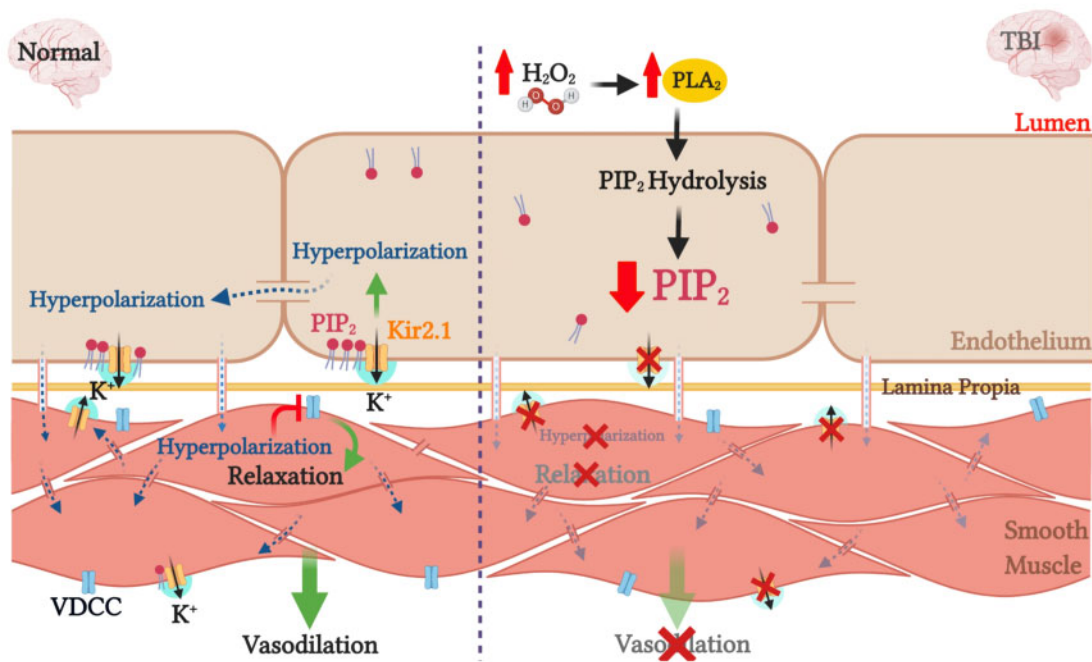*Address correspondence to K.F. (e-mail: kalev.freeman@uvm.edu)

Abstract

Trauma can lead to widespread vascular dysfunction, but the underlying mechanisms remain largely unknown. Inward-rectifier potassium channels (Kir2.1) play a critical role in the dynamic regulation of regional perfusion and blood flow. Kir2.1 channel activity requires phosphatidylinositol 4,5-bisphosphate (PIP_2), a membrane phospholipid that is degraded by phospholipase A_2 (PLA_2) in conditions of oxidative stress or inflammation. We hypothesized that PLA_2-induced depletion of PIP_2 after trauma impairs Kir2.1 channel function. A fluid percussion injury model of traumatic brain injury (TBI) in rats was used to study mesenteric resistance arteries 24 h after injury. The functional responses of intact arteries were assessed using pressure myography. We analyzed circulating PLA_2, hydrogen peroxide (H_2O_2), and metabolites to identify alterations in signaling pathways associated with PIP_2 in TBI. Electrophysiology analysis of freshly-isolated endothelial and smooth muscle cells revealed a significant reduction of Ba^{2+}-sensitive Kir2.1 currents after TBI. Additionally, dilations to elevated extracellular potassium and $BaCl_2$- or ML 133-induced constrictions in pressurized arteries were significantly decreased following TBI, consistent with an impairment of Kir2.1 channel function. The addition of a PIP_2 analog to the patch pipette successfully rescued endothelial Kir2.1 currents after TBI. Both H_2O_2 and PLA_2 activity were increased after injury. Metabolomics analysis demonstrated altered lipid metabolism signaling pathways, including increased arachidonic acid, and fatty acid mobilization after TBI. Our findings support a model in which increased H_2O_2-induced PLA_2 activity after trauma hydrolyzes endothelial PIP_2, resulting in impaired Kir2.1 channel function.

Graphical Abstract

Key words: traumatic brain injury; vascular endothelium; ion channels; metabolopathy

Introduction

Traumatic injury represents the most common cause of death in individuals under 44 years old, with the majority of these fatalities due to traumatic brain injury (TBI).[1] In addition to the primary mechanical injury, systemic inflammation subsequent to trauma contributes to coagulopathy, vascular leak, and multiorgan dysfunction. Vascular endothelial cells (ECs) are especially vulnerable to damage from cellular debris and circulating factors released into the bloodstream after an injury.[2–5] Endotheliopathy in trauma is defined as widespread post-injury disruption of critical endothelial functions including the regulation of microvascular blood flow, barrier integrity, and coagulation. Clinical studies have consistently demonstrated that elevated biomarkers of endothelial injury after severe trauma are important predictors of coagulopathy, multiorgan dysfunction, and death.[6–12] We have previously used a rodent model of TBI to study the effects of injury on blood pressure control and microvascular function, and reported that TBI impairs both blood pressure control and endothelial-dependent vasodilation in systemic resistance vessels by uncoupling endothelial nitric oxide synthase (eNOS).[13,14] Recent advances in systems-level screening and analysis have demonstrated profound metabolopathies occur following hemorrhagic shock, TBI, and burn injury, including pronounced alterations in lipid metabolism which would also be expected to impact blood vessel and endothelial functions.[15–20] Despite these recent advances, there are few actual studies of systemic vascular endothelial function after shock or trauma, and underlying mechanisms which lead to endotheliopathy in trauma remain elusive. Here, we used our established TBI model to study downstream consequences

injury on a critical modulator of vascular function: the inward rectifier potassium channel Kir2.1.

There are 15 members of the inward rectifier potassium (Kir) channel family. The Kir2 family is strong inward rectifier K^+ channels, which are activated by external K^+ and require phosphatidylinositol 4,5-bisphosphate (PIP$_2$) for activity.[21] We and others have provided strong evidence that smooth muscle cells (SMCs) and ECs have Kir2.1 channels.[22–27] Kir2.1 channel function plays a key role in setting arterial resting membrane potential, myogenic tone development, and cerebral blood flow regulation.[28,29] We recently demonstrated impaired capillary EC Kir2.1 function in the cortical hemisphere contralateral to a brain injury; but prior studies have not addressed whether this dysfunction extends to blood vessels in the systemic circulation.[30] PIP$_2$ is a minor membrane phospholipid degraded under conditions of oxidative stress or severe inflammation that affect lipid metabolism. Under these conditions, phospholipases (mainly from the A and C families) catalyze membrane phospholipids to form signaling molecules including inositol trisphosphate, diacylglycerol, and arachidonic acid (AA). Specifically, PIP$_2$ is cleaved by phospholipase A$_2$ (PLA$_2$) at the sn-2 acyl bond, freeing AA, which is subsequently modified by downstream cyclooxygenases and lipoxygenases into prostanoids and eicosanoids including prostaglandins and leukotrienes.[31] Disturbed lipid metabolism in severely injured trauma patients is a strong predictor of clinical outcome.[32] Altered fatty acid metabolism has been demonstrated in controlled animal models of stroke[33] and tissue injury with hemorrhagic shock, including derangements in mono- and poly-unsaturated fatty acid mobilization and oxidation products.[18] We hypothesized

that severe TBI, even without concomitant shock or hypoxia, would cause significant metabolic responses. In this context, depletion of endothelial PIP_2 in the plasma membrane may impair Kir2.1 channel function and therefore, vascular function. Here, we demonstrate a novel mechanism of altered systemic vascular function after TBI, through PIP_2-dependent impairment of Kir2.1 channel function. The physiological stimulus for vascular Kir2.1 channels is elevation of local K^+. Impairment of Kir2.1 channel function is significant, as loss of function disrupts control of regional blood flow to metabolically active microvascular beds. Control of PIP_2 levels may provide a therapeutic target to improve vascular function in conditions characterized by altered lipid metabolisms such as TBI and stroke.

Materials and Methods

Animal and Injury Model

Adult male Sprague-Dawley rats (aged 3–4 months; 300–325 g; Charles River, Saint Constant, Quebec, QC, Canada) were randomly assigned to either a fluid percussion TBI surgery or control treatment, as previously described.[34] All procedures were approved by the Institutional Animal Care and Use Committee and were performed in accord with the National Research Council's *Guide for the Care and Use of Laboratory Animals*. Animals were anesthetized with inhaled 2%–5% isoflurane prior to TBI or sham surgery. We were unable to conceal group allocations due to obvious postsurgical differences. Male animals were used for all studies due to known sexual dimorphism in response to TBI. All animals received buprenorphine analgesia (subcutaneous; 0.05 mg/kg) while under anesthesia and at 6–12 h after surgery. Briefly, a primary injury was induced by a direct contusion to the brain delivered to the left cerebral hemisphere by a pendulum impacting a fluid-filled chamber connected to the intact dura through a craniotomy.[29,30] A fluid percussion injury was induced to a target pressure of 4.7 ± 0.1 atm ($n = 36$; ~70 PSI) over a 500 ms period; the pressure was transduced and measured in each surgery. This level of fluid percussion pressure produces a severe injury with approximately 10% mortality. This is consistent with other publications which have defined "severe injuries" as those produced by pressures >2.8 atm.[35] This level allows >90% recovery, defined as ability to maintain upright posture, ambulate, and take oral hydration, and produces a highly reproducible outcome of moderate brain injury severity in those surviving animals. Animals that did not recover within 60 min were excluded. These experimental animals have measurable deficits in sensorimotor coordination, along with significant cardiovascular and cerebrovascular effects.[29,30] Control animals were subjected to scalp incisions but without the percussion injury. At 24 h after recovery from surgery, animals were euthanized using deep pentobarbital anesthesia (intraperitoneal; 0.03 mg/kg) and tissues were collected for experiments.

Sample Preparation and UHPLC-MS Analysis

Plasma was collected and stored at $-80°C$ until analysis. Analyses were performed as previously published.[36,37] Prior to LC-MS analysis, samples were placed on ice and diluted with 24 volumes of methanol:acetonitrile:water (5:3:2, v:v). Suspensions were vortexed continuously for 30 min at 4°C. Insoluble material was removed by centrifugation at 10 000 g for 10 min at 4°C and supernatants were isolated for metabolomics analysis by

UHPLC-MS. The analytical platform employs a Vanquish UHPLC system (Thermo Fisher Scientific, San Jose, CA) coupled online to a Q Exactive mass spectrometer (Thermo Fisher Scientific, San Jose, CA). Samples were resolved over a Kinetex C18 column, 2.1 × 150 mm, 1.7 μm particle size (Phenomenex, Torrance, CA) equipped with a guard column (SecurityGuard™ Ultracartridge—UHPLC C18 for 2.1 mm ID Columns—AJO-8782—Phenomenex, Torrance, CA) using an aqueous phase (A) of water and 0.1% formic acid and a mobile phase (B) of acetonitrile and 0.1% formic acid for positive ion polarity mode, and an aqueous phase (A) of water:acetonitrile (95:5) with 1 mmol/L ammonium acetate and a mobile phase (B) of acetonitrile:water (95:5) with 1 mmol/L ammonium acetate for negative ion polarity mode. Samples were eluted from the column using either an isocratic elution of 5% B flowed at 250 μL/min and 25°C or a gradient from 5% to 95% B over 1 min, followed by an isocratic hold at 95% B for 2 min, flowed at 400 μL/min and 45°C. The Q Exactive mass spectrometer (Thermo Fisher Scientific, San Jose, CA) was operated independently in positive or negative ion mode, scanning in Full MS mode (2 μscans) from 60 to 900 m/z at 70 000 resolution, with 4 kV spray voltage, 45 sheath gas, 15 auxiliary gas. Calibration was performed prior to analysis using the Pierce™ Positive and Negative Ion Calibration Solutions (Thermo Fisher Scientific. Metabolite assignments, isotopologue distributions, and correction for expected natural abundances of deuterium, ^{13}C, and ^{15}N isotopes were performed using MAVEN (Princeton, NJ).[38]

Electrophysiology

Kir2.1 currents were monitored in freshly isolated ECs and SMCs from third- and fourth-order branches of mesenteric arteries using patch-clamp electrophysiology, as previously described.[39] Briefly, mesenteric arteries were harvested and ECs were isolated in 1.5 mL dissociation solution containing (in mmol/L): 134 KCl, 6 KOH, 10 NaOH, 1.1 $MgCl_2$, 1.8 $CaCl_2$, 5 EGTA, 10 HEPES (pH 7.3), containing 0.5 mg/mL neutral protease and elastase (0.5 mg/mL; Worthington Biochemical Corp., Lakewood, NJ), for 60 min at 37°C. Collagenase (0.5 mg/mL; Worthington Type I) was added to the dissociation solution for three more minutes. Vessels were then washed in Ca^{2+}-free dissociation solution (4°C) for 5–10 min and the solution triturated 10 times through a custom glass Pasteur pipette to release single ECs into the solution. SMCs were isolated in dissociation solution containing 1 mg/mL papain, 0.5 mg/mL dithioerythritol, and 0.5 mg/mL bovine serum albumin for 25 min. Next, 1 mg/mL of collagenase (Worthington Type IV), 0.25 mg/mL elastase, and 0.5 mg/mL Trypsin inhibitor and 100 μM $CaCl_2$ were exchanged in the solution for 10 min. Electrophysiology was performed in either the conventional or perforated whole-cell configuration. Whole-cell currents were amplified using an Axopatch 200B amplifier (Molecular Devices), filtered at 1 kHz, digitized at 10 kHz, and stored on a computer for offline analysis with Clampfit 10.7 software. Patch pipettes were pulled from borosilicate, microcapillary tubes (1.5-mm O.D., 1.17-mm I.D.; Sutter Instruments, Novato, CA), and fire-polished (resistance ~ 4–6 MΩ). Cells were voltage-clamped at a holding V_M of -50 mV and equilibrated for 15 min in a bath solution containing (in mmol/L): 134 NaCl, 6 KCl, 1 $MgCl_2$, 10 glucose, 2 $CaCl_2$, and 10 HEPES (pH 7.4). For the perforated-patch configuration, pipettes were backfilled with a solution containing (in mmol/L): 10 NaCl, 30 KCl, 110 K^+-Aspartate, 1 $MgCl_2$, 10 HEPES (pH 7.2), and 200–250 μg/mL amphotericin B, added freshly on the day of the experiment. For the conventional whole-cell configuration, the

pipette solution was composed of (in mmol/L): 134 KCl, 6 KOH, 10 NaOH, 1.1 $MgCl_2$, 1.8 $CaCl_2$, 5 EGTA, 10 HEPES (pH 7.2). Ba^{2+}-sensitive Kir2 currents were quantified by elevating extracellular $[K^+]$ from 6 to 60 mmol/L via equimolar replacement of NaCl by KCl. A 400-ms voltage-ramp protocol (-140 mV to $+40$ mV) was applied. All experiments were performed at room temperature ($\sim 22^\circ$C). Control EC capacitance was 10.7 ± 0.5 pF and TBI EC capacitance was 11.0 ± 0.6 pF. Control SMC capacitance was 11.2 ± 1.0 pF and TBI SMC capacitance was 14.1 ± 0.7 pF.

Oxidation–Reduction Potential (ORP) Measurements

Redox balance (integrated measure of the balance between total oxidants and reductants) was evaluated in plasma samples obtained from control and TBI rats by measuring the oxidation–reduction potential (ORP), or total oxidizing capacity.[40] Whole blood was collected at the time of euthanasia into an evacuated tube containing heparin and immediately centrifuged (2000 rpm; 4°C). Plasma samples (30 μL) were collected and tested using the RedoxSYS diagnostic platform, consisting of a micro Pt/AgCl combination redox electrode sensor and benchtop analyzer (Aytu Bioscience, Inc., Englewood, CO). Values were recorded in mV after ORP readings were stable for 10 s. The diagnostic platform was calibrated before use and validated in a previous study.[14]

PLA$_2$ Activity Assay

Total PLA_2 activity was measured in plasma samples obtained from control and TBI rats according to manufacturer's instructions (BioVision, San Jose, CA). Briefly, fluorescence readings were taken with a microplate reader every 17 s for 45 min. Activity was calculated from the linear range of the reaction and corrected for volume and time.

Pressure Myography

Pressure myography studies were conducted as previously reported[13] Immediately after euthanasia, a midline laparotomy was performed and the mesentery was dissected out and placed into cold (4°C) physiological saline solution (PSS) with the following composition (in mmol/L): 119 NaCl, 45 KCl, 24 NaHCO$_3$, 1 KH$_2$PO$_4$, 2.5 CaCl$_2$, 1 MgCl$_2$, and 11 glucose (pH 7.4). The mesentery was then pinned out on a dissecting dish and fourth- and fifth-order mesenteric arteries were dissected free from the surrounding adipose and connective tissue. For each experiment, an individual mesenteric artery was cannulated in a pressure myograph (Living Systems Instrumentation, St. Albans, VT) containing oxygenated 20% O$_2$/5% CO$_2$ PSS at 37°C. Intraluminal pressure during the experiment was maintained at 80 mm Hg using a pressure servo system, and blood vessel diameters were measured using edge-detection software coupled to a camera (IonOptix, Westwood, MA). Arteries were equilibrated for 10 min, pressurized, and allowed to develop spontaneous myogenic tone over the course of 30 min, defined as >20% constriction after equilibration and pressurization of vessel. Myogenic tone was significantly higher in the TBI group (37 $\pm$ 2%; $n = 19$; $P < .05$; unpaired t-test) when compared to controls (28 $\pm$ 2%; $n = 19$) as previously reported.[14] The maximal, or passive, diameters obtained in 0-Ca^{2+} PSS with 100 μM diltiazem were not significantly different between control (156 $\pm$ 5 μm; $n = 19$) and TBI groups (161 $\pm$ 9 μm; $n = 19$). The vascular endothelium was considered intact if a dilation of greater than 85% was elicited using the endothelial-dependent vasodilator NS309 (1 μM; Cayman

Chemical Company, Ann Arbor, MI). Arteries that did not develop spontaneous myogenic tone were excluded.

Statistics

Metabolic pathway analysis, PLS-DA, heat mapping, and hierarchical clustering were performed using the MetaboAnalyst 3.0 package (www.metaboanalyst.com).[41] Hierarchical clustering analysis was also performed through the software GENE-E (Broad Institute, Cambridge, MA). GraphPad Prism software (version 6.03; GraphPad Software, La Jolla, CA) was used for X–Y graphing and analysis; values are presented as means $\pm$ standard error of the mean. Differences were considered significant if $P < .05$. Data were tested for normality and a parametric or nonparametric statistical test was subsequently applied. One- or two-way analysis of variance was used for comparisons of multiple group measurements. In a few experimental series, a single control group was used to test multiple hypotheses. To avoid increasing the likelihood of a Type I error, we used the Bonferroni correction to test each individual hypothesis at a significance level determined by $\alpha = 1/m$, where m is the number of comparisons.

Results

TBI Impairs Vasoconstriction to Kir2.1 Blockade and Dilation to Elevated Extracellular Potassium

To assess vascular Kir2.1 channel function, the inhibitors BaCl$_2$ (100 μmol/L; Figure 1A) and ML 133 (20 μmol/L; Figure 1B) were exogenously applied to mesenteric arteries isolated from control and TBI rats. We found that vasoconstrictions to BaCl$_2$ and ML 133 were both significantly decreased in TBI arteries when compared to controls. Next, a concentration–response curve was performed by exchanging the arteriography chamber buffer with increasing steps of extracellular potassium (K$^+$). Dilations to 10 mmol/L extracellular K+ were significantly reduced in MAs after TBI (Figure 1C). Concentrations at and greater than 14 mmol/L K$^+$ constricted MAs in both groups. Interestingly, constrictions at the maximal concentration of 60 mmol/L K$^+$ remained unaltered in TBI animals, suggesting preserved contractility following injury (Figure 1C). These findings suggest that vascular Kir2.1 channel function is crippled following TBI.

Endothelial and Smooth Muscle Kir2.1 Currents Are Significantly Diminished Following TBI and Subsequently Rescued by Exogenous PIP$_2$

We employed patch-clamp electrophysiology in native EC and SMCs from mesenteric arteries from TBI and control animals. First, we utilized a perforated patch-clamp configuration, in which the cytoplasm remains intact, to measure Kir2.1 currents in freshly isolated cells bathed in a high extracellular $[K^+]_o$ solution (60 mmol/L), used to amplify the inward component of Kir2.1 current amplitude. Under these conditions, the K$^+$ equilibrium potential (E$_K$) was -23 mV. We found in both EC and SMC barium-sensitive currents were significantly reduced following TBI (Figure 2A and B). One possible explanation for impaired Kir2.1 currents is the loss of the essential cofactor, PIP$_2$. In support of this, we found the reduction in the Kir2.1 current observed in ECs after trauma was partially restored when the synthetic PIP$_2$ analog PIP$_2$ diC8 (10 μmol/L) was included in the intracellular pipette solution. Using conventional whole-cell configurations, we found current densities in the TBI cells treated with PIP2-diC8 were not significantly different from either the control PIP2-diC8-treated or naïve control groups (Figure 3).
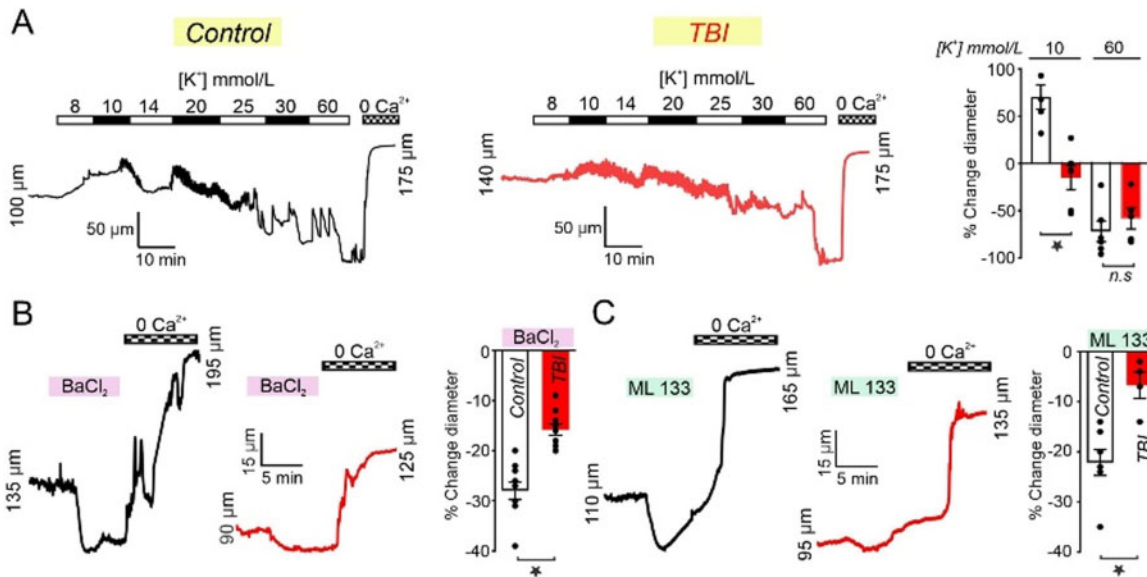
Figure 1. TBI Impairs Vasoconstrictions to the Kir2.1 Channel Blockers BaCl$_2$ and ML 133 and Dilations to Extracellular K$^+$ in Mesenteric Arteries. **(A)** Raising extracellular K$^+$ from 6 to 8 mmol/L caused an immediate $43 \pm 13\%$ ($n = 4$) vasodilation and a subsequent peak dilation at 10 mmol/L K$^+$ ($62 \pm 13\%$; $n = 4$) in control MAs pressurized to 80 mm Hg. As the concentration of extracellular K$^+$ continued to increase from 14 to 60 mmol/L, constrictions were observed in both control and TBI arteries. In MAs from TBI animals responses to 10 mmol/L K$^+$ were significantly diminished when compared to controls ($-15 \pm 13\%$; $n = 6$; $P < 0.05$; unpaired t-test). **(B)** BaCl$_2$ (100 μmol/L)-induced constrictions were significantly diminished in TBI arteries ($-16 \pm 1.1\%$; $n = 10$) when compared to controls ($-28 \pm 1.7\%$; $n = 12$; $P < 0.0001$; unpaired t-test). **(C)** Constrictions to ML 133 (20 μmol/L) were significantly reduced in TBI arteries ($-6.8 \pm 2.6\%$; $n = 4$) when compared to controls ($-22 \pm 2.6\%$; $n = 7$; $P = 0.0091$; unpaired t-test).
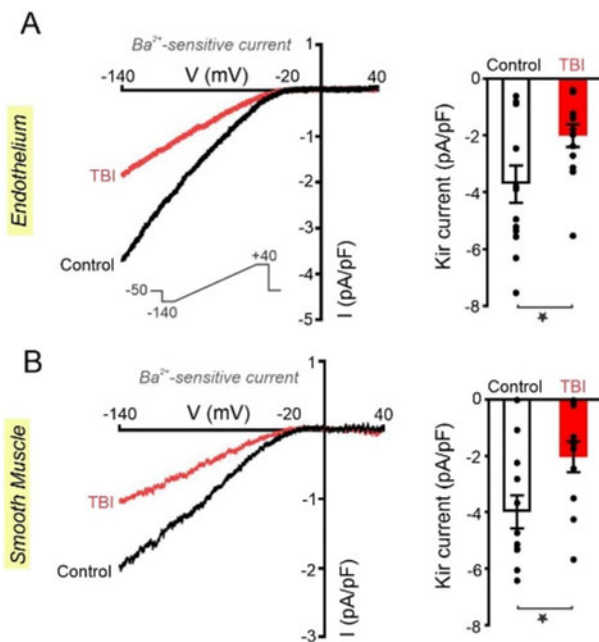

Figure 2. TBI Diminishes Kir2.1 Currents in Mesenteric Endothelial and SMCs. Whole-cell current was measured in freshly isolated endothelial and SMCs in a high extracellular K$^+$ solution (60 mmol/L) using a voltage ramp protocol (-140 to $+40$ mV) and perforated whole-cell patch-clamp configuration, in the absence and presence of BaCl$_2$ (100 μmol/L) **(A)** Representative whole-cell recordings of Ba^{2+}-sensitive currents in ECs from control ($n = 13$) and TBI ($n = 13$) rats. Kir2.1 current density at -140 mV is significantly diminished in ECs from TBI rats when compared to controls (-2.0 ± 0.4 pA/pF, $n = 13$, vs -3.7 ± 0.7 pA/pF, $n = 13$, $P = 0.03$, unpaired t-test). **(B)** Representative whole-cell recording of Ba^{2+}-sensitive currents in SMCs from control ($n = 12$) and TBI ($n = 11$) animals. Similarly, SMC Kir2.1 current density at -140 mV is significantly diminished after TBI when compared to controls (-1.01 ± 0.3 pA/pF, $n = 11$, vs -1.9 ± 0.3 pA/pF, $n = 12$, $P = 0.02$, unpaired t-test; Figure 2B).

TBI Elevates Hydrogen Peroxide-Derived Oxidative Stress and Phospholipase A$_2$ Activity

We previously showed uncoupling of eNOS in systemic arteries after TBI which would be expected to increase vascular hydrogen peroxide (H$_2$O$_2$) levels.[14] Oxidative stress is known to activate PLA$_2$ and contribute to the deranged lipidome observed after TBI.[42–46] We therefore sought to measure global oxidant levels in plasma, and the response of mesenteric arteries to catalase, after TBI. We measured oxidation–reduction potential in plasma before and after the addition of catalase (500 U/mL). The oxidation–reduction potential after TBI was significantly higher compared to controls and was reduced to a level equal to controls by catalase (Figure 4A). This indicates that the overall state of oxidation is elevated in TBI plasma, and that H$_2$O$_2$ is the reactive oxidant species responsible for this elevation. In pressurized mesenteric arteries, constrictions to catalase (500 U/mL) were significantly increased following TBI (Figure 4B). In addition, total PLA$_2$ activity (mU/mL) was significantly increased in plasma from injured animals when compared to controls (Figure 4C). These findings support a model of H$_2$O$_2$-derived PLA$_2$ activity which would be expected to alter lipid metabolism and PIP$_2$ levels after TBI.

TBI Disrupts Lipid Metabolism Causing Accumulation of PIP$_2$ Degradation Products

A high-throughput, semi-targeted metabolomics approach was applied to evaluate the metabolome of rats in our model of TBI, which has been shown to induce endothelial dysfunction in remote blood vessels from the mesenteric circulation.[14] A correlation matrix and heat map for the top 50 metabolites with the lowest P-values studied in plasma from control and TBI rates are shown (Figure 5A and B). Individual metabolites may be seen in Supplementary Table S1. Partial least squares discriminant analysis (PLS-DA) of control and TBI groups of metabolite
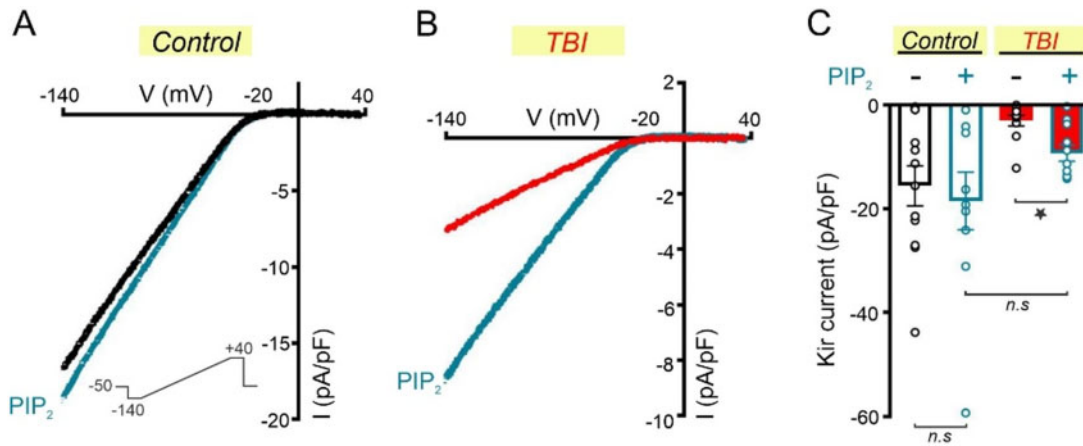
Figure 3. The PIP_2 Analog PIP_2 diC8 Rescues Endothelial Kir2.1 Currents after TBI. Whole-cell currents were measured in freshly isolated endothelial and SMCs in a high extracellular K^+ solution (60 mmol/L) using a voltage ramp protocol (-140 to $+40$ mV) and conventional whole-cell patch-clamp configuration, in the absence and presence of $BaCl_2$ (100 µmol/L). The PIP_2 analog PIP_2 diC8 (10 µmol/L) was included in the patch pipette. (**A**) Representative whole-cell recording of Ba^{2+}-sensitive currents from control and TBI MAs in the absence or presence of PIP_2-diC8 (10 µmol/L). (**B**) Summary data of peak inward current (-140) in ECs dialyzed with or without PIP_2 from control or TBI groups. Kir2.1 current density was significantly diminished after TBI in MA ECs when compared to control ECs without PIP_2 (-2.9 ± 1.0 pA/pF, $n = 11$ vs -15.7 ± 3.8 pA/pF, $n = 12$; $P < 0.0062$; unpaired t-test). No difference was observed in Kir2.1 current density (-140 mV) in the presence or absence of PIP_2 in ECs from control MAs (-18.5 ± 5.5 pA/pF, $n = 10$, vs -15.7 ± 3.8 pA/pF, $n = 12$; n.s.; unpaired t-test). Kir2.1 current density was significantly increased in TBI ECs in the presence of PIP_2 when compared to TBI ECs without PIP_2 (-8.6 ± 1.6 pA/pF, $n = 12$, vs -2.9 ± 1.0 pA/pF, $n = 11$; $P < 0.0078$; unpaired t-test). There was no significant difference in Kir2.1 current density in control and TBI ECs in the presence of PIP_2, implying a rescue of functional Kir2.1 channels and a return to control-level current densities in the ECs from TBI MAs.

levels in each biological replicate showed that the trauma and control samples clustered in distinct metabolic phenotypes (Figure 5C). Fold-change comparisons between TBI and control animals are shown as graphs of lipid metabolites. Of particular interest, we noted that TBI produced profound alterations in certain lipids, including increases in circulating eicosatetraenoic acid and AA, which can derive from PIP_2 degradation. Downstream products of AA metabolism were also altered, including prostanoids, eicosanoids, and leukotrienes (Figure 5D).

Discussion

Here, we used a well-characterized model of TBI, known to produce systemic effects of increased blood pressure and impaired endothelial-dependent vasodilatory function, to study the role of Kir2.1 in the endotheliopathy of trauma. This study has three relevant findings. First, TBI cripples systemic vascular Kir2.1 channel function, evident in both single cell (EC and SMC) and intact vascular preparations. This has important clinical implications, as vascular Kir2.1 signaling modulates blood flow.[39] Second, we provide a plausible mechanism for trauma-induced endothelial dysfunction; an increase in circulating and cellular H_2O_2-derived oxidative stress which causes an increase in PLA_2 activity that would be expected to degrade PIP_2 and thereby altering Kir2.1 channel function. Third, the fact that Kir2.1 channel function in isolated cells was rescued with PIP_2 suggests a potential therapeutic strategy to improve endothelial function, providing a rationale for future experiments that will determine the efficacy of PIP_2 treatment in trauma models. Additionally, we demonstrate that an isolated TBI, even without shock or hypoxia, produces a profound lipidopathy and an increase in H_2O_2 levels in both plasma and small blood vessels. A metabolomics screen was conducted to determine metabolic alterations in our TBI model which might impact vascular functions. This probe identified dysregulation of lipid metabolism pathways, including alteration of arachidonate pathways and

phospholipid synthesis, and fatty acid mobilization/oxidation which are hallmarks of inflammation. Increases in H_2O_2-derived oxidative stress and phospholipase activity in plasma were consistent with upregulation of lipid metabolism.[19,42,45,47] We focused on PIP_2, a specific membrane phospholipid and key modulator of Kir2.1, the molecular feature that regulates myogenic tone and electrical conduction through the vascular endothelium.[28,48–51]

Endotheliopathy in Trauma

Recent studies have reported that biomarkers of endothelial dysfunction correlate with injury severity and adverse outcomes including coagulopathy and multiorgan dysfunction in trauma patients. Biomarkers reflecting endothelial damage or activation by cytokines include syndecan-1, thrombomodulin, and E-selectin.[7,8,10,52] These biosignatures are elevated after isolated TBI and/or multisystem injury, in adults and children, and in both the prehospital setting and after resuscitation. These reports have suggested the existence of a pervasive "endotheliopathy of trauma" which complicates recovery and prevents optimizing the management of uncontrolled hemorrhage and coagulopathy; yet few studies have actually evaluated EC or vascular function directly after trauma. Animal models of trauma provide the opportunity to directly study blood vessels to further elucidate the cellular mechanisms of endothelial injury. We previously described abnormal blood pressure control and impaired endothelial-dependent vasodilation in blood vessels harvested from the mesenteric circulation, remote from the site of TBI, but, to our knowledge, the function of vascular Kir2.1 channels in trauma has not been previously assessed.[13,14] Here, we provide a novel mechanism for endothelial dysfunction, showing that Kir2.1 function in pressurized arteries and isolated vascular cells is significantly diminished; thus, providing a novel model to elucidate dysfunction in the endotheliopathy of trauma.
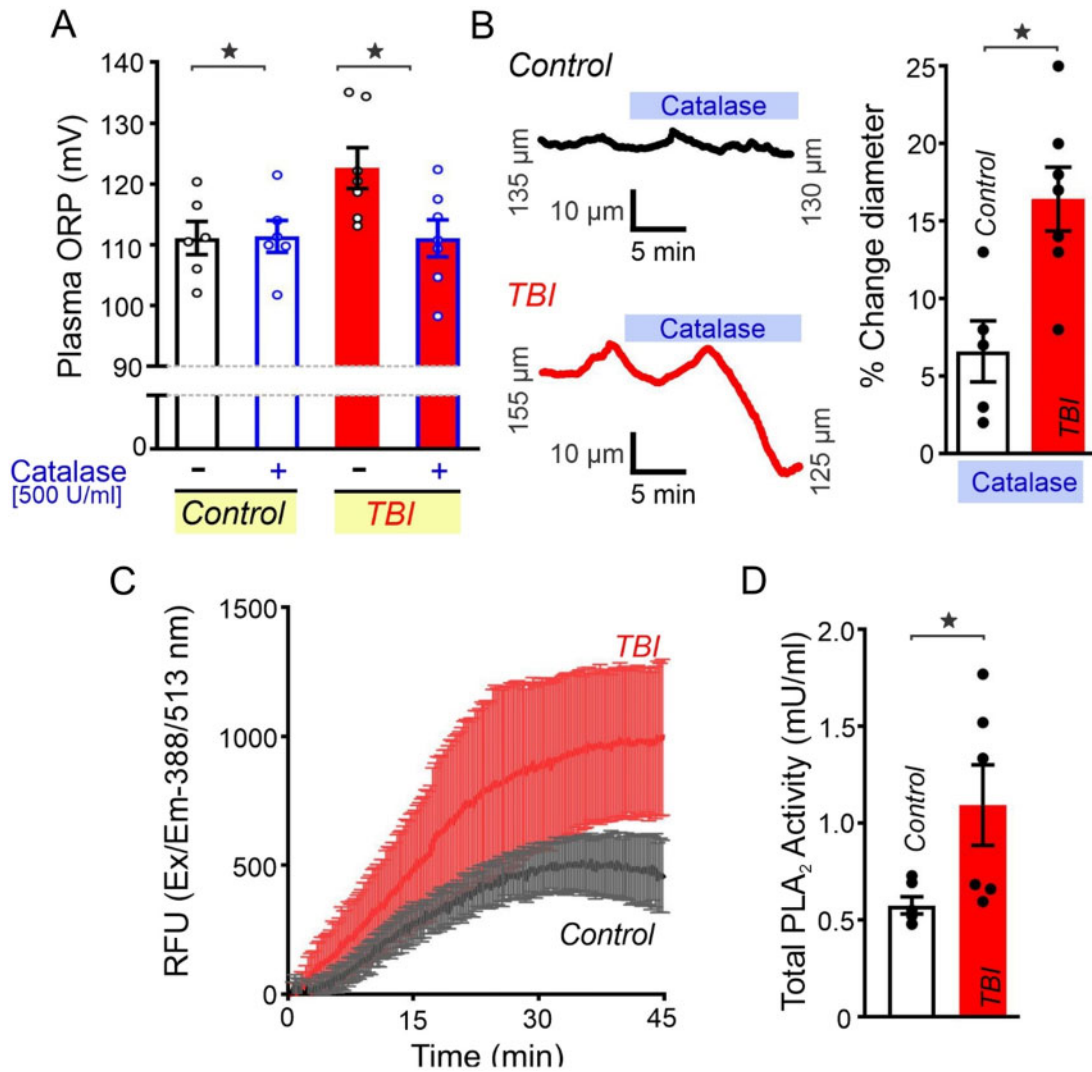
Figure 4. H_2O_2 Levels and PLA_2 Activity Are Significantly Increased in Mesenteric Arteries and Plasma from TBI rats. (**A**) Oxidative reduction potential (ORP) measurements using the Redoxsys platform were conducted in paired plasma samples from control and TBI rats with and without the addition of catalase (500 U/mL). Plasma ORP was unchanged in control samples before and after the addition of catalase (500 U/mL) (111.1 ± 2.7 mV vs 111.4 ± 2.6 mV, $n = 6$; n.s.; paired t-test). ORP in TBI plasma samples was significantly decreased to control values after the addition of catalase (500 U/mL) (122.6 ± 3.6 mV vs 111.1 ± 3.0 mV, $n = 7$, $P = 0.0017$, paired t-test). (**B**) Constrictions to catalase (500 U/mL) were significantly increased in mesenteric arteries from TBI rats when compared to controls ($16.4 \pm 2.01\%$, $n = 7$ vs $6.6 \pm 2.0\%$, $n = 5$, $P = 0.0077$; unpaired t-test). (**C**) Averaged time course of PLA_2 activity in control and TBI plasma samples. (**D**) Total PLA_2 activity is significantly increased in plasma from TBI rats when compared to controls (1.095 ± 0.21 mU/mL, $n = 6$ vs 0.576 ± 0.05 mU/mL, $n = 6$, $P = 0.0352$; unpaired t-test).

Kir2.1 Channel Dysfunction and PIP_2 Regulation

The Kir2.1 channel is the molecular cornerstone responsible for dynamic regulation of blood flow in the brain and other excitable tissues, and the membrane phospholipid PIP_2 is required for channel function.[21] Kir2.1 channels are exquisitely sensitive to changes of extracellular K^+, which acts as a potent vasodilator. In the brain, capillary endothelial Kir2.1 channels play a critical role in the dynamic regulation of regional perfusion, by conducting a spreading hyperpolarizing signal in response to locally elevated extracellular K^+ released during neural activity, and thereby directing blood flow.[28] We demonstrated that the electrical response of brain capillary endothelial Kir2.1 and its ability to direct blood flow depends on PIP_2 levels.[51] Cerebral ischemia leads to a loss of functional Kir2.1 channels in parenchymal arteriole smooth muscle, impairing neurovascular coupling.[53] In addition, others have investigated the effect of

fluid percussion injury on ATP-sensitive potassium channels and calcium-activated potassium channels in the cerebral vasculature.[54–56] Our current study is novel as we elucidate the effects of TBI on EC inward rectifier potassium channels in a remote, systemic tissue bed. We recently found that a TBI results in impaired Kir2.1 channel function in capillary ECs from the opposite side of the brain, and we hypothesized that this dysfunction may be widespread and not limited to cerebral endothelium. Endothelial Kir2.1 channels are also important in mesenteric resistance arteries, boosting vasodilatory signals through endothelial-derived hyperpolarizing pathways.[48] Our results support a model by which systemic oxidative stress, involving H_2O_2, acts via PLA_2 to disrupt Kir2.1 function (Figure 6). Our model provides a plausible mechanism by which the metabolic stress of trauma leads to systemic endothelial dysfunction, based on the known link between H_2O_2 and PLA_2 activation.[42,47,57,58]
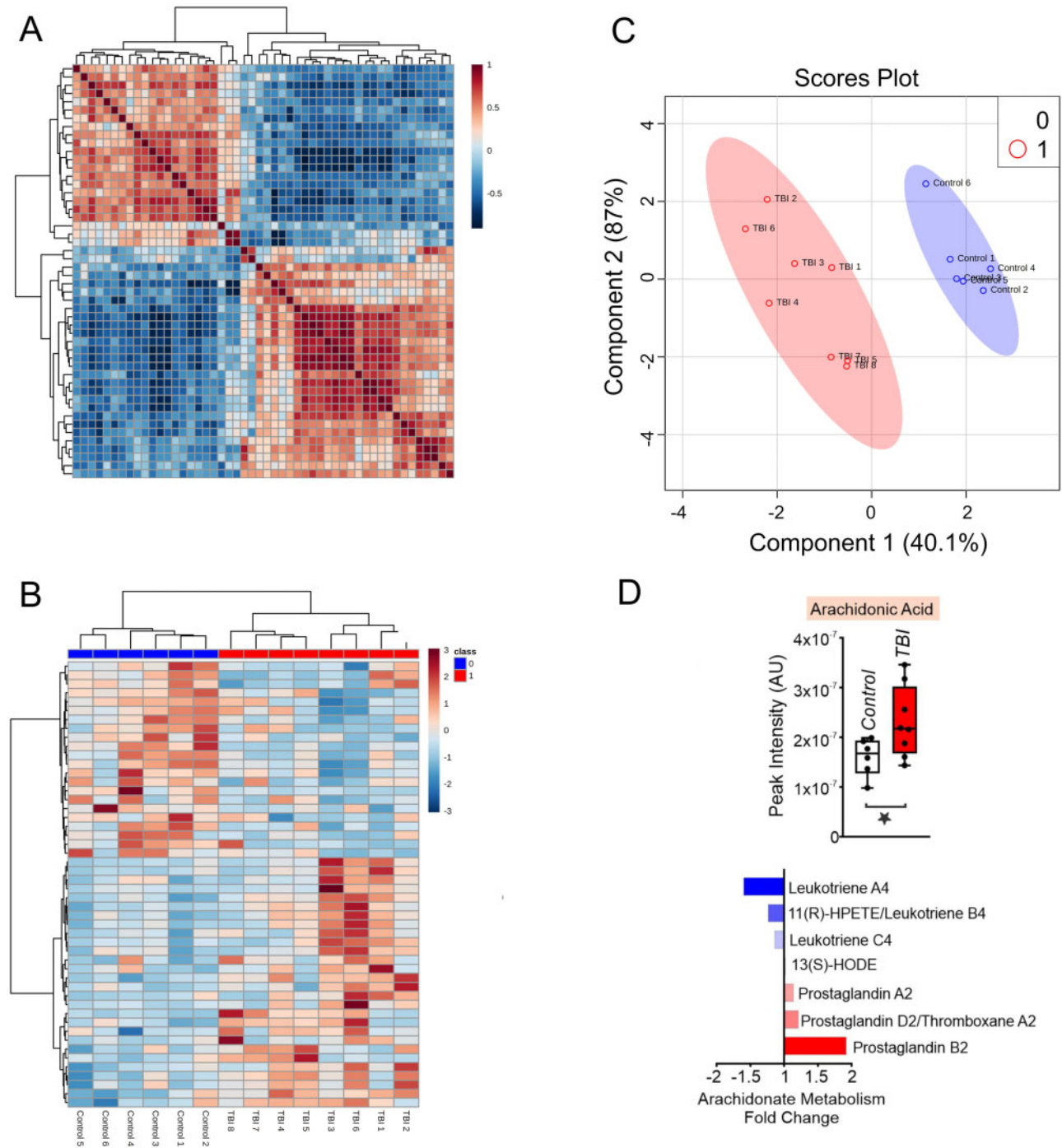
Figure 5. TBI Causes Accumulation of PIP$_2$ Degradation Products and a Profound Lipidopathy. (**A**) Correlation matrix (distance measure: Pearson *r*) of the top 50 metabolites sorted by lowest *P*-value and (**B**) a heat map (distance measure: Euclidean; clustering algorithm: Ward) of the same dataset. (**C**) Scores plot with Component 1 on the *x*-axis contributes to 40.1% of the total variance. Component 2 on the *y*-axis contributes to 87% of the total variance. (**D**) Circulating AA (eicosatetraenoic acid) is significantly increased in plasma from TBI rats when compared to controls (2.3×10^7 median AU, $n = 8$ vs 1.6×10^7 median AU, $n = 6$, $P < 0.05$). Arachidonate metabolism pathway fold changes after TBI.

Deranged Lipid Metabolism, H$_2$O$_2$, and PLA$_2$ after TBI

Providing further support for our model, our metabolomics screen demonstrated that lipid metabolism homeostasis is significantly deranged as early as 1 day after TBI. These findings correlate with prior reports on TBI during the subacute and chronic phases suggesting that disturbed lipid metabolism is a strong predictor of clinical outcome.[18,32] With the aid of mass spectrometry-based lipidomics, they provided quantitative characterization of lipid peroxidation products after TBI and acknowledge PLA$_2$ as an important mediator in the hydrolysis of peroxidized phospholipids.[58] The polyunsaturated fatty acid (PUFA) component of membrane phospholipids are often targets for peroxidation, broadly defined as the process of inserting a hydroperoxy group into a lipid. Peroxidation increases the
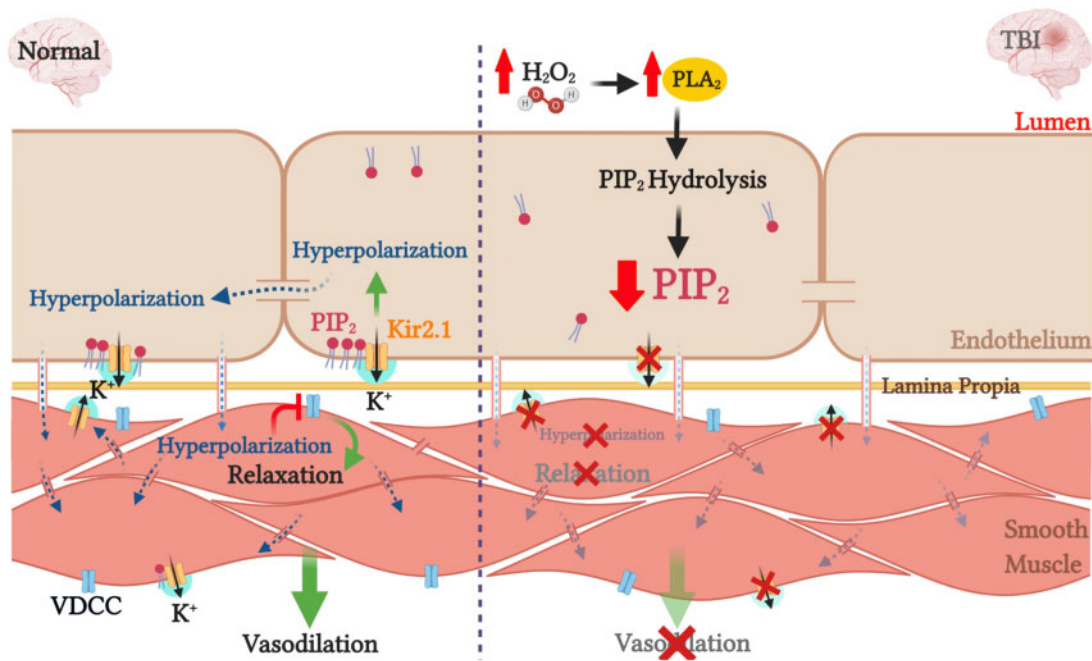
Figure 6. Schematic Overview. As PIP_2 is required to stabilize Kir2.1 channel activity, and H_2O_2 drives down PIP_2 levels, we hypothesized that TBI results in impaired Kir2.1 channel function in MAs. We demonstrated that 24 h following TBI an increase in circulating and cellular H_2O_2-derived oxidative stress causes an increase in PLA_2 activity which degrades PIP_2 in the cell membrane, thereby impairing Kir2.1 channel function and subsequent hyperpolarization/vessel dilation.

rates of hydrolysis by phospholipases. The extent of lipid peroxidation after severe TBI correlates with injury severity and mortality.[58] Rodent models have also provided insight into specific changes in lipid metabolism induced by trauma. Major changes in free fatty acids, including docosahexaenoic, stearic, oleic, and AAs, were sustained from 4 to 35 days after injury in a rat.[59] In addition, significant upregulation in PUFAs and PUFA-containing diacylglycerols and changes in membrane phospholipids including sphingolipids have been observed at 1 week after injury.[17] Lipidomic analyses in mouse model of TBI identified injury-specific phospholipid changes that persisted as long as 3 months after trauma induction.[15] Our findings showed that not only lipid metabolites were altered after TBI, but also plasma H_2O_2 and PLA_2 activity were significantly increased in these animals. Specific metabolites of PLA_2-dependent PIP_2 degradation products, such as AA and its downstream metabolites, were effectively detected in our model of TBI. In this context, increased oxidative stress has been described after severe trauma and in TBI in particular.[60]

Conclusions

Collectively, our results support a novel mechanism to explain endotheliopathy in trauma, through diminished Kir2.1 responses which can be rescued by PIP_2. These findings may be generalizable to other pathological conditions characterized by altered lipid metabolism pathways and reductions in PIP_2 levels, and suggest PIP_2 may be a potential therapeutic target to improve endothelial function in conditions characterized by altered lipid metabolism such as brain trauma and stroke. Further investigations are warranted to determine the relevant time course along with the potential for sexual dimorphism in Kir2.1 channel function after TBI. In addition, experiments to explore the effect of plasma H_2O_2, sPLA2, and altered lipid metabolism on vascular Kir2.1 function in which animals are pretreated

with PEG-Catalase or sPLA2 inhibitors prior to surgery would provide additional evidence to support our model and could inform future therapeutic strategies.

Supplementary Material

Supplementary material is available at the *APS Function* online.

Authors' Contributions

A.M.S. designed experiments, acquired, and analyzed data, wrote the initial draft, and edited all subsequent drafts of the manuscript. N.V. assisted with experimental design and participated in all stages of critical revisions. A.D.B. acquired and analyzed electrophysiology data. A.D. and T.M. acquired and analyzed mass spectrometry data. M.S. contributed to data representation and in critical revisions of the manuscript. O.F.H. participated in critical revisions of the manuscript. M.T.N. and K.F. directed the study and edited the manuscript. All authors reviewed the manuscript, contributed to critical revisions, and approved its submission.

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Conflict of interest statement

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