Inhibition of Aryl Hydrocarbon Receptor Attenuates Hyperglycemia-Induced Hematoma Expansion in an Intracerebral Hemorrhage Mouse Model

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BACKGROUND: Hyperglycemia is associated with greater hematoma expansion (HE) and worse clinical prognosis after intracerebral hemorrhage (ICH). However, the clinical benefits of intensive glucose normalization remain controversial, and there are no approved therapies for reducing HE. The aryl hydrocarbon receptor (AHR) has been shown to participate in hyperglycemia-induced blood–brain barrier (BBB) dysfunction and brain injury after stroke. Herein, we investigated the role of AHR in hyperglycemia-induced HE in a male mouse model of ICH.

METHODS AND RESULTS: CD1 mice (n=387) were used in this study. Mice were subjected to ICH by collagenase injection. Fifty percent dextrose was injected intraperitoneally 3 hours after ICH. AHR knockout clustered regularly interspaced short palindromic repeat was administered intracerebroventricularly to evaluate the role of AHR after ICH. A selective AHR inhibitor, 6,2′,4′- trimethoxyflavone, was administered intraperitoneally 2 hours or 6 hours after ICH for outcome study. To evaluate the effect of AHR on HE, 3-methylcholanthrene, an AHR agonist, was injected intraperitoneally 2 hours after ICH. The results showed hyperglycemic ICH upregulated AHR accompanied by greater HE. AHR inhibition provided neurological benefits by restricting HE and preserving BBB function after hyperglycemic ICH. In vivo knockdown of AHR further limited HE and enhanced the BBB integrity. Hyperglycemia directly activated AHR as a physiological stimulus in vivo. The thrombospondin-1/transforming growth factor-β/vascular endothelial growth factor axis partly participated in AHR signaling after ICH, which inhibited the expressions of BBB-related proteins, ZO-1 and Claudin-5.

CONCLUSIONS: AHR may serve as a potential therapeutic target to attenuate hyperglycemia-induced hematoma expansion and to preserve the BBB in patients with ICH.

Key Words: blood ■ hyperglycemia ■ intracerebral hemorrhage
Ren et al AHR Mediates Hematoma Expansion After ICH

HE, little is known regarding the effect of hyperglycemia on HE and blood–brain barrier (BBB) dysfunction following ICH. Moreover, despite being nondiabetic, 44.7% of patients with acute ICH later presented with new-onset hyperglycemia,7 but the clinical benefit of intensive glucose normalization remains controversial. The GISK-UK (glucose insulin in stroke trial, United Kingdom), trial of insulin to control blood sugar after acute stroke using magnetic resonance imaging (MRI) end-points (SELESTIAL), and the stroke hyperglycemia insulin network effort (SHINE) are 3 large randomized controlled trials with the objective of targeting post-stroke hyperglycemia with insulin infusion, which failed to provide benefits to patients with stroke.6 These studies indicate that interventions to counter the deleterious effect of hyperglycemia-related HE should be explored through other underlying mechanisms.

Aryl hydrocarbon receptor (AHR) is a ligand-activated transcription factor located in the cytoplasm.9 AHR is known to be widely expressed in astrocytes, endothelial cells, and neuronal cells in the brain,9 and it plays important roles in sensing and metabolizing xenobiotic factors such as polyaromatic hydrocarbons and environmental toxins with its highly conserved PER-ARNT-SIM domain.11 As demonstrated by the phenotype in AHR knockout mice,12 AHR was postulated to be linked with vascular and cardiac homeostasis, immune system function, and neoplasm development in response to endogenous ligands such as kynurenine and tryptophan metabolite under normal cell physiology. The pathophysiological roles of AHR in the central nervous system have been extensively studied.10 In experimental stroke models, inhibition of AHR was demonstrated to have neuroprotective effects,13 but the mechanisms underlying BBB protection remain poorly explored. Furthermore, there are no studies that have investigated the role of AHR in HE after ICH. A recent study showed that AHR was activated in response to high glucose stimulation, which led to the formation of a complex with 2 other glucose-sensitive transcription factors, Egr-1 and AP-2.14 The active AHR complex was shuttled into the nucleus and activated the glucose-responsive gene promoter fragment of thrombospondin-1 by binding to it. Furthermore, the thrombospondin-1/transforming growth factor-β (TGF-β)/vascular endothelial growth factor (VEGF) pathway has been implicated in BBB breakdown.15

Herein, we explored the role of AHR on hematoma expansion after ICH. We investigated the effects of AHR inhibition on hematoma expansion and explored underlying mechanisms of protection pertaining to BBB protection after hyperglycemic ICH in mice (Figure S1).

METHODS

Data Availability
All data are available within the article and additional data can be acquired from the corresponding author.

Reagents
The AHR antagonist, 6,2′,4′-trimethoxyflavone (TMF), AHR agonist, 3-methylcholanthrene (3-MC), dimethyl sulfoxide (DMSO), dextrose, and mannitol were from Sigma-Aldrich, MO. LSKL, Thrombospondin (TSP-1) inhibitor, was from MedChem Express, NJ.

Animal Models and Experimental Design
A total of 387 adult male CD-1 mice (weight 30–35 g, Charles River, Wilmington, MA) aged 8–10 weeks were used for the study. All mice were housed under a 12-hour light/dark cycle with ad libitum access to food and standard water. All animal experimental procedures were approved by the Institutional Animal Care and Use Committee.
Committee at Loma Linda University (#8190039) and were compliant with the Animal Research: Reporting In Vivo Experiments (ARRIVE) guidelines. Mice were randomly assigned to different experimental groups by generating random numbers. Information on experimental groups was blinded to researchers who performed surgeries, neurobehavioral assessments, Western blot, immunofluorescence staining, and data analysis. Six separate experiments were conducted (Figure S2). Animal numbers per group are listed in Table S1.

**Experiment 1. To Investigate the Effect of Hyperglycemia on HE after ICH**
Forty-two mice were assigned to 3 groups: sham, ICH, and ICH+dextrose. Blood glucose and hematoma volume were measured accordingly. Additionally, 6 mice were used to evaluate the impact of hyperosmotic solution on hematoma expansion in the ICH+mannitol group.

**Experiment 2. To Evaluate Time Course and Cellular Localization of AHR in the Ipsilateral Hemisphere After ICH**
Sixty mice were assigned to the following 9 groups: sham, ICH (6h), ICH(12h), ICH(24h), ICH(72h), ICH+dextrose(6h), ICH+dextrose(12h), ICH+dextrose(24h) and ICH+dextrose(72h). Western blot was performed to evaluate temporal changes in protein expressions, and double immunofluorescence staining was used to explore cellular localization of AHR after ICH.

**Experiment 3. To Assess the Effect of Treatment with AHR Antagonist TMF on HE, BBB Damage, and Neurobehavioral Outcomes After Hyperglycemic ICH**
Part 1. One hundred sixteen mice were divided into 7 groups: sham, ICH, ICH+dextrose, ICH+dextrose+DMSO, ICH+dextrose+TMF(low dose), ICH+dextrose+TMF(middle dose, 5mg/kg), ICH+dextrose+TMF(high dose, 15mg/kg). Hematoma volume, neurobehavioral tests, and brain water content were measured at 24 hours and 72 hours after ICH. Tail bleeding time and clot formation ex vivo were also measured. Additionally, 6 mice were used to evaluate neurobehavioral tests and hematoma expansion in the ICH+TMF group.
Part 2. Sixty-four mice were divided into the following 5 groups: sham, ICH, ICH+dextrose, ICH+dextrose+DMSO, and ICH+dextrose+TMF (5 mg/kg). BBB permeability was evaluated using IgG staining, Evan’s blue extravasation, double immunofluorescence staining, and neurobehavioral tests were measured.

**Experiment 4. To Determine the Role of AHR on HE and BBB Function After Hyperglycemic ICH**
Sixty-six mice were assigned to the following 4 groups: Sham, ICH+dextrose, ICH+dextrose+AHR clustered regularly interspaced short palindromic repeat (CRISPR) knockout, and ICH+dextrose+knockout CRISPR Control. Western blots, hemoglobin assay, Evan’s blue extravasation, double immunofluorescence staining, and neurobehavioral tests were measured.

**Experiment 5. To Explore Hyperglycemia Interaction with AHR and HE After ICH**
Thirty-six mice were assigned to 3 groups: ICH, ICH+dextrose, and ICH+3-MC. Immunoprecipitation was performed to detect the interaction between AHR with Egr-1.

**Experiment 6. To Determine Potential Deleterious Molecular Mechanism of AHR-Mediated BBB Disruption and HE Expansion After Hyperglycemic ICH**
Fifty-four mice were assigned to 9 groups: sham, ICH+dextrose, ICH+dextrose+DMSO, ICH+dextrose+TMF, ICH+dextrose+TMF+CRISPR Control, ICH+dextrose+TMF+thrombospondin-1 CRISPR activation, ICH+dextrose+3-MC, ICH+dextrose+3-MC+DMSO, and ICH+dextrose+3-MC+LSKL. Western blots were used to evaluate the protein expression changes.

**Collagenase-Induced ICH Model and Hyperglycemia Procedure**
The collagenase injection ICH model was performed as described previously. Briefly, the mice were weighed and anesthetized with a ketamine–xylazine cocktail (1.5 mL/kg). Next, the animals were fixed to a stereotactic frame in a prone position and the position of the Hamilton syringe was adjusted to ensure proper injection site relative to bregma (right lateral 2.2 mm, rostral 0.2 mm). A burr hole was drilled at the position. Next, the infusion pump was set at a rate of 0.2 μL/min and the needle of the Hamilton syringe was advanced ventrally to a depth of 3.5 mm to infuse 1 μL collagenase (0.075 U). The needle was left in situ for 5 minutes after the end of infusion before retracting at a rate of 1 mm/min. Sham group mice were subjected to a similar procedure but received 0.9% sterile saline injection. Three hours after ICH induction, the mice were injected with 6 mL/kg of 50% dextrose intraperitoneally. Baseline and follow-up blood glucose levels up to 8 hours postsurgery were measured using a glucometer (ReliOn) in tail vein blood.
Neurobehavioral Tests
The modified Garcia neurological score, corner turn test, forelimb placement test, and Rotorod test were used to assess neurobehavioral function.\textsuperscript{18}

Brain Water Content Measurement
Wet–dry weight measurement for brain edema was performed as previously reported.\textsuperscript{19} Brain water content (%) was calculated as (wet weight–dry weight)/wet weight*100%.

Intracerebroventricular Injection
As previously described,\textsuperscript{20} intracerebroventricular injection in the left ventricle was performed using the coordinates left lateral of bregma=1.0 mm and ventral depth=3.2 mm. For each of the CRISPRs, a total of 2 \( \mu \)L CRISPR (final concentration 0.5 \( \mu \)g/\( \mu \)L) was injected into the left lateral ventricle 48 hours before ICH. The detailed procedure is shown in Data S1.

Western Blots and Immunoprecipitation Assay
Western blots and immunoprecipitation assay were performed as reported previously.\textsuperscript{21} The antibodies used are listed in Table S2. Western blot bands were quantified as relative density of bands using Image J software (NIH, Bethesda, USA).

Hemoglobin Assay and Image-Based Measurement of Hematoma Volume
A modified spectrophotometric assay was used to measure hemoglobin content, and a step-by-step procedure for hematoma volume measurement was followed as per a previously published protocol.\textsuperscript{22}

Tail Bleeding Monitoring and Ex Vivo Thrombus Formation
Hemostasis was assessed by measuring bleeding time using the tail transection test as described previously.\textsuperscript{23} Details of the procedure are shown in Data S1.

Evan's Blue Dye Extravasation
Evan’s blue extravasation was used to evaluate BBB disruption as reported before.\textsuperscript{24} The optical density was measured at 610 nm.

Immunohistochemistry
Immunofluorescence staining was performed on frozen brain sections as reported previously.\textsuperscript{21} The antibodies used are listed in Table S2. Immunohistochemistry staining for IgG was performed using VECTASTAIN ABC Kit (Peroxidase-HRP, PK-4002, VECTOR Laboratories).

Veriloid's staining was conducted using IRON STAIN Kit (HT 20, Sigma-Aldrich) following the procedure suggested by the manufacturer. All the morphological results were photographed using a Zeiss microscope equipped with a digital color camera.

Statistical Analysis
All data analysis was performed using GraphPad Prism 8.2.1 (GraphPad Software, CA) and data were expressed as mean±SD. Multiple comparisons were statistically analyzed with Kruskal–Wallis test followed by post hoc Dunn’s method or 2-way ANOVA followed by the post hoc Tukey method. Statistical significance was defined as \( P < 0.05 \).

RESULTS

Hyperglycemia Exacerbated Hematoma Expansion After ICH
The hyperglycemic ICH group had a significantly larger hematoma compared with the ICH–only group (\( P < 0.05 \), Figure 1A through 1C). TMF did not have any effect on plasma glucose levels. Hyperosmotic 20% mannitol did not significantly affect HE (Figure S3B and S3C).

AHR Was Upregulated and Expressed in Astrocytes and Endothelial Cells After Hyperglycemic ICH
Temporal AHR expression after hyperglycemic ICH was upregulated at \( \approx 6 \) hours and peaked at 24 hours (\( P < 0.05 \), Figure 1D and 1E). AHR mainly localized in perivascular astrocytes as well as in endothelial cells after hyperglycemic ICH (Figure 1F).

TMF Attenuated HE, and Improved Short- and Long-Term Outcomes in Hyperglycemic ICH Mice

TMF Limited HE and Improved Neurobehavioral Function
TMF was administered in a dose-gradient manner 2 hours after ICH. TMF 5 mg/kg significantly attenuated HE, and exhibited better neurobehavioral function and less brain water content at 24 hours after hyperglycemic ICH. Given these findings, only the 5 mg/kg dose of TMF was used to evaluate outcomes at 72 hours and for rest of the experiments. Seventy-two hours after hyperglycemic ICH, TMF 5 mg/kg improved neurobehavioral function, attenuated HE, and alleviated BWC in ipsilateral basal ganglia and cortex (\( P < 0.05 \), Figure 2A and 2B).
Delayed TMF administration (5 mg/kg administered at 6 hours after ICH) also significantly limited HE and improved neurological function at 24 hours after hyperglycemic ICH (P<0.05, Figure 2C). Additionally, TMF had no effect on hemostasis in vivo and ex vivo (Figure S4C).

**TMF Alleviated BBB Impairment and Improved Long-Term Neurobehavioral Outcome**

Hyperglycemic ICH mice receiving TMF (5 mg/kg) demonstrated a lesser extent of BBB leakage and ferric iron deposition around the perihematomal region in basal ganglion (P<0.05, Figure 3A through 3C, and 3E). Likewise, TMF 5 mg/kg improved long-term sensorimotor and balance function at 1 to 3 weeks after ICH (Figure 3D).

**Effect of AHR Knockout in Hyperglycemic ICH Mice**

To verify in vivo AHR CRISPR knockout efficacy, mice were subjected to AHR CRISPR knockout, which showed significantly decreased expression of AHR as well as downstream proteins thrombospondin-1, TGF-β, and VEGF while ZO-1 was upregulated compared with control CRISPR (P<0.05, Figure 4A) in vivo AHR CRISPR knockout ameliorated HE and preserved BBB disruption, thus improving neurobehavioral function.
after hyperglycemic ICH (P<0.05, Figure 4B through 4D).

**Hyperglycemia-Activated AHR and Aggravated HE After ICH**

The interaction of AHR with Egr-1 was significantly increased in hyperglycemic ICH mice compared with sham and ICH mice (P<0.05, Figure 5A and 5B). ICH mice subjected to either dextrose or 3-MC induced a similar severity of HE and BBB leakage (Figure 5C through 5E).

**AHR Modulated BBB Dysfunction Through Activation of Thrombospondin-1/TGF-β/VEGF Signaling Pathway**

The temporal expression patterns of thrombospondin-1, TGF-β, and VEGF, as AHR-related signaling downstream pathway proteins, were consistent with the time course expression of AHR (Figure S5).

At 24 hours after hyperglycemic ICH, TMF significantly decreased the expression of AHR, thrombospondin-1, TGF-β, and VEGF, whereas ZO-1 and Claudin-5 protein expression increased. Thrombospondin-1
CRISPR activation significantly reversed the aforementioned protein expression except for AHR, which showed no such changes (P<0.05, Figure 6A and 6B). Likewise, 3-MC augmented the expression of thrombospondin-1, TGF-β, VEGF, and diminished ZO-1, Claudin-5, and these changes were reversed with LSKL (P<0.05, Figure 6C and 6D).

**DISCUSSION**

Since the first clinical account of HE was observed and documented in 1937, clinicians have long sought aggressive intervention for HE. Hyperglycemia is associated with greater HE and poorer clinical outcomes after ICH. However, it is more important to demonstrate causation from mechanism to effect rather than correlation. AHR emerged initially as a novel class of protein, an orphan receptor that also functions as a transcription factor with important roles in maintaining physiological functions for the immune system, neurological system, and barrier organs. The objective of this study was to explore whether AHR played a role in hyperglycemia-induced HE, and whether AHR inhibition ameliorates HE after experimental hyperglycemic ICH in mice. Here, we showed that hyperglycemia exacerbated early HE after ICH. Hyperglycemic ICH induced AHR intracellular overexpression primarily in astrocytes and endothelial cells, which participated in HE concomitantly with upregulation of VEGF-mediated BBB disruption. We also reported that high glucose may account for the direct activation of AHR in this setting. To the best of our knowledge, the present study was the first to demonstrate a pathophysiological role of AHR in ICH as well as the first ever to report glucose as a physiological stimulus for AHR in vivo, revealing a potential therapeutic target from the bench to bedside.

Approximately 50% of patients with acute onset of stroke may develop stress-induced hyperglycemia even in the absence of preexisting diabetes, and emerging evidence demonstrates that during admission to the hospital, stress-induced hyperglycemia was associated with early HE and mortality. To mimic stress-induced hyperglycemia and to improve
the clinical relevance of this study, we gave a single dose of dextrose injection to CD-1 mice 3 hours after ICH onset, which was in accordance with the average time interval from symptom onset to hospital admission or randomization. Other research teams have also explored the effect of hyperglycemia on HE. One study compared the hemorrhagic response between streptozotocin-induced diabetic and nondiabetic rats using an autologous blood injection model by quantifying hematoma enlargement using the subarachnoid space expansion as an indicator. Another study injected collagenase to induce ICH and monitored temporal hematoma formation by magnetic resonance imaging in streptozotocin-treated rats. Regardless of the differences in subject species and high glucose patterns in these studies, our results were consistent with previous studies, which revealed that a greater macroscopic hematoma and increased bleeding volume were observed in the right basal ganglion as early as 6 hours after hyperglycemic ICH. The hematoma volume peaked at 24 hours and plateaued until 72 hours, whereas plasma glucose returned to baseline long before by 8 hours after ICH. Therefore, we highlighted hyperglycemia as a
triggering factor for HE acting independently of other variables.

Our understanding regarding the scope of AHR-modulated biological and pathophysiological processes has continued to expand, since they were first discovered 2 decades ago. Several studies reported that the expression of AHR was increased during the early and subacute phases of both ischemic and traumatic brain injuries. AHR was detected in the human cerebral microvascular endothelial cell line and astrocytes isolated from the murine BBB. To the best of our knowledge, the present study was the first to show that the AHR signaling pathway was induced in astrocytes and endothelial cells after a deleterious insult such as hemorrhagic stroke.

The early profiles of AHR activation and transcriptional activity strongly suggest a detrimental role of the receptor in the hemorrhagic pathophysiological process. Administration of the specific AHR antagonist TMF limited hematoma enlargement and elicited better neuroprotective outcomes in a dose-dependent manner in hyperglycemic ICH mice. Conversely, 3-MC, a prototypical AHR agonist, aggravated HE and BBB permeability in ICH mice. Taken together, these findings indicated that AHR played a role in HE after hyperglycemic ICH. The specific AHR antagonist such as TMF may be useful for inhibiting detrimental actions of the receptor after hyperglycemic ICH. However, regulatory roles of AHR in brain pathogenesis are sometimes controversial, which in part may be dependent on the different pharmacological properties of AHR ligands. The partial agonist of AHR, 3,3′-diindolylmethane, may exert an anti-inflammatory effect via activating AHR, and the therapeutic effects of laquinimod, with AHR-activating property, on brain pathology may also be mediated mainly by AHR, although the contribution of AHR-independent actions was not excluded. In line with these previous studies, we found an uptrend of AHR expression between 12 and 72 hours after ICH in mice without dextrose insult, though there was no
significant difference between the 2 interventions (ICH versus ICH+TMF) in terms of hematoma volume and short-term outcomes at 24 hours after ICH (Figure S4A and S4B). Further research is warranted for other potential novel endogenous ligands of AHR in response to ICH.

As a crucial pathophysiological feature following ICH, BBB disruption has been considered to be closely involved in HE. However, it remains under debate as to whether the relationship between BBB and HE after ICH is causative or reciprocal. This study demonstrated that hyperglycemic ICH mice exhibited degradation of ZO-1 and Claudin-5, increased extravasation leakage of Evan’s blue and IgG, and overexpression of VEGF, which indicated more severe BBB damage. Experimental animals as well as human studies have led to the hypothesis that BBB dysfunction may trigger HE after ICH. A systematic review discussing BBB...
dysfunction in the pathogenesis of ICH because of cerebral small vessel disease found that there was a temporal relationship between BBB dysfunction and cerebral hemorrhage.\textsuperscript{36} BBB disruption markers such as tight junction protein degradation, leakage of contrast agent, plasma protein extravasation, and serum VEGF level indicated vascular injury\textsuperscript{36} and predicted subsequent HE.\textsuperscript{37} We found that AHR inhibition using ischemia, suggesting an anti-apoptosis effect of AHR element-binding protein survival/death signaling after pharmacological modulation.\textsuperscript{13} Additionally, several injection to induce ICH, does not emulate the cause of spontaneous ICH in clinical scenarios such as hypertensive vasculopathy and cerebral amyloid angiopathy. Given the challenges inherent in animal models, more studies are necessary to explore the clinical impact regarding the role of AHR in hyperglycemic ICH. Also, we only used young male mice in this study. The rationale for choosing young and male mice was to rule out confounding factors, in this case, sex and age. Given the possibility that there might be different therapeutic and side effects in old or female mice, further studies are warranted to determine sex- and age-dependent effects. Second, we used CRISPR to edit the in vivo genome. Although control CRISPR was also used to exclude potential off-target effects, there may still be some unpredictable off-target effects on neurobehavioral function and protein expression. For further validation of the AHR function, transgenic animals should be considered. Lastly, although this study found that the mechanism of action for AHR was derived from downstream BBB dysfunction, AHR has been shown to exhibit anti-apoptotic effect after ischemic stroke.\textsuperscript{13} Therefore, further studies are necessary to elucidate other roles and mechanisms of AHR in ICH.

CONCLUSIONS

The present study showed that hyperglycemia increased the magnitude of HE during experimental ICH, and this effect was mediated by AHR. A hyperglycemia-sensitive mechanism for AHR-mediated impairment of BBB integrity could contribute to hematoma expansion after ICH. Overall, this study linked hyperglycemia with AHR signaling in ICH and identified AHR as a potential therapeutic target in this setting.

ARTICLE INFORMATION

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Disclosures

None.

Supplementary Material

Data S1
Tables S1–S2
Figures S1–S5
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SUPPLEMENTAL MATERIAL
Data S1.

Supplemental Materials & Methods

Intracerebroventricular injection
Using clustered regularly interspaced short palindromic repeat/Cas9 (CRISPR/Cas9) technology, we edited the in vivo genome mediated by homology-independent targeted integration. We used AHR CRISPR/Cas9 KO Plasmid (sc-419054, Santa Cruz) to inhibit AHR expression in the mouse brain and TSP-1 CRISPR Activation Plasmid (sc-423381-ACT, Santa Cruz) to activate TSP-1 expression in the mouse brain. The CRISPR 20μg was suspended in 20μL of transfection medium (sc-108062, Santa Cruz) and then activated using 20μL transfection reagent (sc-395739, Santa Cruz) to get a final concentration 0.5 μg/μL of CRISPR. For each of the CRISPRs, a total of 2μL CRISPR was injected into the left lateral ventricle 48h before ICH.

Tail Bleeding Monitoring
Hemostasis was assessed by measuring bleeding time using the tail transection test. Tail transection (at 2 mm tail diameter) was performed 30 minutes after the end of collagenase, vehicle (DMSO), or TMF administration, and the bleeding times (visual observation) were recorded.

Ex Vivo Thrombus Formation
A modified ex vivo clot formation model was used. In brief, three groups of mice were used here, namely ICH+Dx+DMSO, ICH+Dx+TMF, and ex vivo TMF groups. For mice in DMSO and ex vivo TMF groups, whole blood was harvested by cardiac puncture, and 200 μL of the blood was mixed and vortexed with 600 μL of DMSO ex vivo or 600 μL of 100 μM TMF ex vivo. For mice in the ICH+Dx+TMF group, 800 μL of whole blood was harvested via cardiac puncture. Samples were kept at room temperature for 2 minutes, followed by centrifugation for 30 seconds at 4,000 g. The tubes were gently removed and the amount of uncoagulated supernatant was quantified.
Table S1. Animal use and mortality rate

| Group                        | Mortality | Subtotal |
|------------------------------|-----------|----------|
| **Experiment 1**             |           |          |
| Sham                         | 0% (0/6)  | 6        |
| ICH (6, 24, 72h)             | 0% (0/18) | 18       |
| ICH+Dx (6, 24, 72h)          | 0% (0/18) | 18       |
| ICH+Mannitol 24h             | 0% (0/6)  | 6        |
| **Experiment 2**             |           |          |
| Sham                         | 0% (0/8)  | 8        |
| ICH (6, 12, 24, 72h)         | 0% (0/26) | 26       |
| ICH+Dx (6, 12, 24, 72h)      | 0% (0/26) | 26       |
| **Experiment 3**             |           |          |
| Sham                         | 0% (0/24) | 24       |
| ICH                          | 0% (0/12) | 12       |
| ICH+TMF                      | 0% (0/6)  | 6        |
| ICH+Dx                       | 3.7% (1/26+1) | 27       |
| ICH+Dx+DMSO                  | 5.3% (2/36+2) | 38       |
| ICH+Dx+TMF (1.5mg/kg)        | 7.7% (1/12+1) | 13       |
| ICH+Dx+TMF (5mg/kg)          | 0% (0/50) | 50       |
| ICH+Dx+TMF (15mg/kg)         | 0% (0/12) | 12       |
| **Experiment 4**             |           |          |
| Sham                         | 0% (0/18) | 0        |
| ICH+Dx                       | 5.2% (1/18+1) | 0        |
| ICH+Dx+AH CRISPR KO          | 0% (0/18) | 18       |
| ICH+Dx+CRISPR Control        | 14.3% (2/12+2) | 14       |
| **Experiment 5**             |           |          |
| Sham                         | 0% (0/12) | 0        |
| ICH                          | 0% (0/18) | 6        |
| ICH+Dx                       | 0% (0/18) | 0        |
| ICH+3-MC                     | 14.3% (2/12+2) | 14       |
| **Experiment 6**             |           |          |
| Sham                         | 0% (0/6)  | 0        |
| ICH+Dx                       | 0% (0/6)  | 0        |
| ICH+Dx+DMSO                  | 0% (0/6)  | 6        |
| ICH+Dx+TMF                   | 14.3% (0/6) | 6        |
| ICH+Dx+TMF+CRISPR Control    | 0% (0/6)  | 6        |
| ICH+Dx+TMF+TSP-1 CRISPR Activation | 14.3% (1/6+1) | 7        |
| ICH+Dx+3-MC                  | 14.3% (1/6+1) | 7        |
| ICH+Dx+3-MC+DMSO             | 14.3% (1/6+1) | 7        |
| ICH+Dx+3-MC+LSKL             | 0% (0/6)  | 6        |
| **Total**                    | 3.1%      | 387      |
Table S2. Antibodies used in Western blot and Immunohistochemistry

| Species and Name                              | Dilution rate | Manufacture           |
|-----------------------------------------------|---------------|-----------------------|
| Rabbit polyclonal Anti-AHR antibody           | 1:500         | ab84833, Abcam         |
| Rabbit polyclonal Anti-TSP-1 antibody         | 1:1000        | ab85762, Abcam         |
| Rabbit polyclonal Anti-TGF-β antibody         | 1:1000        | ab92486, Abcam         |
| Rabbit polyclonal Anti-VEGFA antibody         | 1:1000        | ab46154, Abcam         |
| Rat monoclonal Anti-ZO-1 antibody             | 1:1000        | sc-33725, Santa Cruz   |
| Rabbit monoclonal Anti-Claudin 5 antibody     | 1:1000        | ab131259, Abcam        |
| Mouse monoclonal Anti-β-actin                 | 1:3000        | sc-47778, Santa Cruz   |
| Mouse monoclonal Egr-1 antibody               | -             | sc-515830, Santa Cruz  |
| Protein A/G PLUS- Agarose                     | -             | sc-2003, Santa Cruz    |
| Anti-glial fibrillary acidic protein           | 1:100         | Santa Cruz             |
| Anti-VWF antibody                             | 1:100         | Santa Cruz             |
The effects of AHR inhibition on hematoma expansion and underlying mechanisms of protection pertaining to BBB protection after hyperglycemic ICH in mice. The cell type in representative figure is astrocyte. AHR, aryl hydrocarbon receptor; ICH, intracerebral hemorrhage; BBB, blood-brain barrier; TMF, trimethoxyflavone; CRISPR, clustered regularly interspaced short palindromic repeat; KO, knockout; TSP-1, thrombospondin-1; TGF-β, transforming growth factor-β; VEGF, vascular endothelial growth factor; ZO-1, zonula occludens-1; EC, endothelial cells; TJ, tight junction.
ICH, intracerebral hemorrhage; Dx, dextrose; HE, hematoma expansion; AHR, aryl hydrocarbon receptor; BBB, blood-brain barrier; DMSO, dimethyl sulfoxide; TMF, trimethoxyflavone; CRISPR, clustered regularly interspaced short palindromic repeat; KO, knockout;
(A) Plasma glucose measured using glucometer by tail-tip transection in different groups. Sham group was shared between 6h, 24h and 72h. The error bars represent mean ± SD. * P<0.05 vs Sham. # P<0.05 vs ICH. n=6 for each group. Two-way ANOVA, Tukey’s test. (B-C) Statistical analysis of hematoma volume and hemoglobin assay in ICH, ICH+Mannitol and ICH+Dx group at 24h after ICH. The error bars represent mean ± SD. # P<0.05 vs ICH. n=6 for each group. Kruskal-Wallis test, Dunn’s test. ICH, intracerebral hemorrhage; Dx, dextrose; TMF, trimethoxyflavone.
Figure S4. TMF had no effect on neurobehavioral function, hematoma volume and hemostasis in ICH mice at 24h.

(A-B) Statistical analysis of modified garcia test, forelimb placing, corner turn test and hematoma volume in Sham, ICH, ICH+TMF group at 24h after ICH. (C) Tail bleeding time in different groups 30 min after administration. Un-coagulated content measurement ex vivo in different groups. The error bars represent mean ± SD. * P<0.05 vs Sham. ns, no statistical difference. n=6 for each group. Kruskal-Wallis test, Dunn’s test. ICH, intracerebral hemorrhage; DMSO, dimethyl sulfoxide; TMF, trimethoxyflavone.
(A) Representative and quantitative analysis (B-D) of western blot bands showing temporal expressions of TSP-1, TGF-β and VEGF at 6h, 12h, 24h, 72h in ICH group and ICH+Dx group. Sham group was shared between 6h, 24h, 72h. The error bars represent mean±SD. * P<0.05 vs Sham. # P<0.05 vs ICH. Two-way ANOVA, Tukey’s test. ICH, intracerebral hemorrhage; Dx, dextrose; TSP-1, thrombospondin-1; TGF-β, transforming growth factor-β; VEGF, vascular endothelial growth factor.