17β-Estradiol Attenuates Hematoma Expansion Through Estrogen Receptor α/Silent Information Regulator 1/Nuclear Factor-kappa B Pathway in Hyperglycemic Intracerebral Hemorrhage Mice

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Background and Purpose—17β-estradiol (E2) has been reported to reduce bleeding and brain injury in experimental intracerebral hemorrhage (ICH) model. However, it is not clear if E2 can prevent early hematoma expansion (HE) induced by hyperglycemia in acute ICH. The aim of this study is to evaluate the effects of E2 on HE and its potential mechanisms in hyperglycemic ICH mice.

Methods—Two hundred, 8-week-old male CD1 mice were used. ICH was performed by collagenase injection. 50% dextrose (8 mL/kg) was injected intraperitoneally 3 hours after ICH to induce acute HE (normal saline was used as control). The time course of HE was measured 6, 24, and 72 hours after ICH. Two dosages (100 and 300 μg/kg) of E2 were administered 1 hour after ICH intraperitoneally. Neurobehavioral deficits, hemorrhage volume, blood glucose level, and blood–brain barrier disruption were measured. To study the mechanisms of E2, estrogen receptor α (ERα) inhibitor methyl-piperidino-pyrazole, silent information regulator 1 (Sirt1) siRNA was administered, respectively. Protein expression of ERα, Sirt1, and acetylated nuclear factor-kappa B, and activity of matrix metalloproteinases-9 were detected.

Results—Hyperglycemia enhanced HE and deteriorated neurological deficits after ICH from 6 hours after ICH. E2 treatment prevented blood–brain barrier disruption and improved neurological deficits 24 and 72 hours after ICH. E2 reduced HE by activating its receptor ERα, decreasing the expression of Sirt1, deacetylation of nuclear factor-kappa B, and inhibiting the activity of matrix metalloproteinases-9. ERα inhibitor methyl-piperidino-pyrazole and Sirt1 siRNA removed these effects of E2.

Conclusions—E2 treatment prevented hyperglycemia-enhanced HE and improved neurological deficits in ICH mice mediated by ERα/Sirt1/nuclear factor-kappa B pathway. E2 may serve as an alternative treatment to decrease early HE after ICH. (Stroke. 2015;46:485-491. DOI: 10.1161/STROKEAHA.114.006372.)

Key Words: 17β-Estradiol ■ intracerebral hemorrhage ■ MMP-9 ■ Sirt1

Spontaneous intracerebral hemorrhage (ICH) is a subtype of stroke featured by hematoma formation within brain parenchyma, which accounts for ≈15% of all deaths from stroke and with >75% of patients severely disabled or deceased within the first year.¹ The high mortality and morbidity make ICH a major public health problem, and no effective therapy has yet been established to counteract the consequences of this detrimental subtype of stroke subtype. After ICH, the initial hematoma forms and is untreatable. However, ≈30% of ICH patients continue to bleed and demonstrate significant hematoma expansion (HE), which further aggravates the outcome.² Most HE occurs within 3 hours after the onset of ICH³ and is amenable to treatment.⁴ Because of its strong relationship with poor prognosis and the potential to prevent its development, HE is an appealing therapeutic target after ICH.

Estrogens are steroid compounds that function as the primary sex hormone in females, and 17β-estradiol (E2) is the most potent naturally occurring estrogen. In the last 2 decades, estrogen is one of the most extensively studied neuroprotectants for the treatment of stroke.⁵⁻⁶ In experimental ICH model, estrogens have been proven to reduce bleeding and brain injury in rats,⁷⁻¹¹ whereas the mechanisms underlying blood–brain barrier (BBB) protection of E2 remain poorly explored.
and there are no studies to investigate the effects of estrogens on early HE after ICH. Matrix metalloproteinases (MMPs), which are regulated by nuclear factor-kappa B (NF-κB), have been proved to be the culprit for BBB disruption. Recently, E2 has been reported to upregulate silent information regulator 1 (Sirt1), which can inactivate NF-κB. In the present study, we investigate whether E2 treatment will prevent the early growth of HE induced by hyperglycemia in ICH mice and explore the potential role of Sirt1/NF-κB in BBB protection.

Materials and Methods
All experiments were approved by the Institutional Animal Care and Use Committee of Loma Linda University.

Animal Model and Experimental Protocol
Two hundred, 8-week-old male CD1 mice (weight 25–35 g; Charles River, Wilmington, MA) were used. ICH mouse model was performed by collagenase injection as reported previously.50% dextrose (8 mL/kg) was injected intraperitoneally 3 hours after ICH to induce acute HE (normal saline was used as control). The time course of HE was measured 6, 24, and 72 hours after ICH. Two dosages (100 and 300 μg/kg) of E2 (Sigma-Aldrich) were administrated 1 hour after ICH intraperitoneally. Neurobehavioral deficits, hemorrhage volume, and blood glucose were measured. To study the mechanisms of E2, estrogen receptor α (ERα) inhibitor methyl-piperidino-pyrazole (MPP; Sigma-Aldrich; 100 μg/kg) and Sirt1 siRNA (Origene Technologies) was administered, respectively. Protein expression of ERα, Sirt1, deacetylated NF-κB, and activity of MMP-9 were detected. The experimental design was included in Figure 1 in the online-only Data Supplement.

siRNA Injection
Two different formats of Sirt1 siRNA were applied 48 hours before ICH to enhance the silencing effect. An intracerebroventricular injection was then performed as previously described.14 The Sirt1 siRNA or scramble siRNA mixed with the transfection reagent (Origene Technologies; 100 pmol/2 μL) was delivered into the ipsilateral ventricle with a Hamilton syringe and administered over 2 minutes. After the needle was removed, the burr hole was sealed with bone wax. The incision was closed with sutures, and the mice were allowed to recover.

Neurological Scores
Twenty-four or 72 hours after ICH, the Garcia test was performed by a blinded investigator as previously described with modifications.15 The scores given to each of the mice at the completion of the evaluation was the summation of 7 individual test scores (spontaneous activity, symmetry in the movement of 4 limbs, forepaw outstretching, climbing, body proprioception, response to vibrissae touch, and beam walking). The neurological scoring ranged from 2 (most severe deficit) to 21 (maximum).

Hematoma Volume
Hemoglobin assay was performed as previously described.14 The ipsilateral hemisphere was homogenized for 60 seconds in a tube with distilled water (total volume 3 mL). After centrifuging (12,000 g, 30 minutes), Drabkin’s reagent (400 μL; Sigma-Aldrich) was mixed in with a supernatant (100 μL) and allowed to react for 15 minutes. The absorbance of the mixture was read with a spectrophotometer (540 nm), and the amount of blood in each brain was calculated using a standard curve generated with known blood volumes.

Evans’s Blue Dye Extravasation
Disruption of the BBB was analyzed 24 hours after ICH using Evans’s blue dye as reported previously.15 The amount of extravasated Evans’s blue in the brain was determined by spectrophotometry with a standard curve. Measurements were conducted at an excitation wavelength of 610 nm.

Immunohistochemistry
Immunofluorescent staining for brain tissue was performed on fixed frozen ultrathin sections as previously described. Primary antibodies used were ERα (SAB4500810; Sigma-Aldrich), Sirt1 (S5447; Sigma-Aldrich), glial fibrillary acidic protein (sc-6170; Santa Cruz Biotechnology), and Von Willebrand factor (sc-8068; Santa Cruz Biotechnology). Peri-hemorrhagic area of the brain coronal section was imaged by Olympus-BX51.

Western Blot Analysis
Brain samples were collected 24 hours after the ICH. Western blotting was performed as described previously. Primary antibodies used were ERα (SAB4500810; Sigma-Aldrich), Sirt1 (S5447; Sigma-Aldrich), acetylated NF-κB (SAB4502616; Sigma-Aldrich), and β-actin (sc-1616; Santa Cruz Biotechnology).

Matrix Metalloproteinase Zymography
The ipsilateral brain cortex was used to analyze MMP-9. Briefly, samples were homogenized, and the supernatant was collected. Samples were loaded and separated by 10% Tris-tricine gel with 0.1% gelatin as a substrate. After separation by electrophoresis, the gel was renatured and then incubated with development buffer at 37°C for 24 hours. The gel was stained with 0.5% coomassie blue R-250 for 30 minutes and then destained appropriately. MMP-9 activity was quantified using ImageJ, version 1.32.

Statistical Analysis
The analysis of the data was performed using GraphPad Prism software. Statistical differences between 2 groups were analyzed using the student’s unpaired, 2-tailed t test. Multiple comparisons (without a rating scale) were statistically analyzed with 1-way analysis of variance followed by the Tukey method. The data are presented as means±SEM. In all statistical analysis, a value of P<0.05 represents statistical significance.

Results
Hypercglycemia Enhanced HE and Deteriorated Neurobehavioral After ICH
To observe the effects of hyperglycemia on HE, we administrated saline or 50% dextrose (8 mL/kg) to the animals 3 hours after ICH and measured the hemorrhagic volume and neurobehavioral at 6, 24, and 72 hours after ICH. There was distinct hematoma formation at the basal ganglia from 6 hours after collagenase injection. In ICH+saline group, the hemorrhagic volume 6, 24, and 72 hours were 19.29±1.917, 18.89±1.367, and 13.40±1.056 μL, respectively. Hyperglycemia significantly enhanced HE to 29.70±3.368, 31.87±1.972, and 27.40±2.100 μL, respectively (Figure 1A and 1B; P<0.05 versus ICH+saline) and deteriorated neurological deficits (Figure 1C; P<0.05 versus ICH+saline). Blood glucose peaked 1 hour after DX injection and returned to baseline within 4 hours, and E2 had no effect on blood glucose level (Figure 1D; P>0.05 versus ICH+DX).

E2 Suppressed HE and Improved Neurological Deficits 24 and 72 Hours After ICH
A low dosage of E2 showed the tendency to attenuate the HE. A high dosage of E2 significantly decreased hemorrhagic volume (Figure 2A and 2B; P<0.05 versus ICH+DX+vehicle). This dosage of E2 (300 μg/kg) significantly improved the
neurological scores compared with vehicle group 24 hours after ICH (Figure 2C; *P<0.05 versus ICH+DX+vehicle). The beneficial effects of E2 on BBB disruption and neurological deficits last ≤72 hours after ICH (Figure 3; P<0.05 versus ICH+DX+vehicle).

**ERα Inhibitor MPP and Sirt1 siRNA Abolished the Effects of E2 24 Hours After ICH**

To investigate the potential mechanisms of E2 in suppressing HE, we administrated ERα inhibitor MPP 45 minutes after ICH and Sirt1 siRNA 48 hours before ICH, respectively, with the treatment of a high dosage of E2. MPP significantly increased the hemorrhagic volume from 21.97±1.11 to 29.47±1.99 μL (Figure 4A and 4B; *P<0.05 versus ICH+DX+E2) and decreased the neurological scores from 13.17±1.22 to 9.25±0.49 24 hours after ICH (Figure 4C; *P<0.05 versus ICH+DX+E2). Sirt1 siRNA also abolished the effects of E2 by increasing the hemorrhagic volume to 28.25±0.68 μl (Figure 4A and 4B; &P<0.05 versus ICH+DX+E2) and decreased the neurological scores from 13.17±1.22 to 9.75±0.62 (Figure 4C; *P<0.05 versus ICH+DX+E2). These data suggested that E2 ameliorated HE dependent on its receptor ERα and Sirt1.
E2 Ameliorated HE by Inhibiting the Activity of MMP-9 Through ERα/Sirt1/NF-κB Pathway

Double fluorescence immunostaining showed the expression of ERα and Sirt1 were strong in astrocytes and endothelial cells in sham animals and decreased after ICH (Figure 5). Western blots showed that after ICH, there was a dramatic loss of ERα in ipsilateral brain tissue (Figure 6A; P<0.05 versus Sham), and administration of E2 significantly upregulated the expression of ER-α (Figure 6A; P<0.05 versus ICH+DX+vehicle), whereas ERα inhibitor MPP did not affect the expression of ERα significantly (Figure 6A; P>0.05 versus ICH+DX+E2). The expression of Sirt1 decreased after ICH (Figure 6B; P<0.05 versus Sham) and increased by E2 24 hours after ICH (Figure 6B; P<0.05 versus ICH+DX+vehicle). Sirt1 siRNA strongly knocked down the expression of Sirt1 in both Sham and ICH mice compared with scramble siRNA (Figure II in the online-only Data Supplement). MPP and Sirt1 siRNA abrogated the results of E2 and decreased the expression of Sirt1 (Figure 6B; P<0.05 versus ICH+DX+E2). ICH increased the acetylation of NF-κB (Figure 6C; P<0.05 versus Sham), which was decreased by E2 administration (Figure 6C; P<0.05 versus ICH+DX+vehicle). Administration of MPP or Sirt1 siRNA increased the acetylated NF-κB in E2-treated animals (Figure 6C; P<0.05 versus ICH+DX+E2).

The activity of MMP-9 in ICH+DX+vehicle group was intensely increased 24 hours after ICH (Figure 6D; P<0.05 versus Sham) and was significantly decreased by E2 (Figure 6D; P<0.05 versus ICH+DX+vehicle). ER-α inhibitor

Figure 3. E2 prevented hematoma expansion (A and B), improved Garcia neuroscores (C), and preserved the integrity of blood–brain barrier (BBB; D) 72 h after intracerebral hemorrhage (ICH). *P<0.05 vs sham; & P<0.05 vs ICH+DX+vehicle. n=6 for hematoma expansion and Garcia neuroscores in each group; n=5 for Evan’s blue in each group.

Figure 4. Estrogen receptor α (ERα) inhibitor methyl-piperidino-pyrazole (MPP) and Sirt1 siRNA removed the effects of E2 on hematoma expansion (A, B) and Garcia neuroscores (C) 24 hours after intracerebral hemorrhage (ICH). *P<0.05 vs sham, & P<0.05 vs ICH+DX+vehicle, # P<0.05 vs ICH+DX+E2. n=6 for each group.
MPP and Sirt1 siRNA abolished the effects of E2 on the activity of MMP-9 (Figure 6D; \( P < 0.05 \) versus ICH+DX+E2). The activity of MMP-2 did not show great changes in all groups. The results suggested that E2 inhibited the activity of MMP-9 through ER\( \alpha \)/Sirt1/NF-\( \kappa \)B pathway in ICH mice.

**Discussion**

Hematoma volume is a major factor of both mortality and poor outcome after ICH. Early HE aggravates initial outcomes and increase mortality. Early HE is, however, treatable, and restriction of HE is a promising therapeutic approach. In this study, our goals were to test, first, whether hyperglycemia enhanced early HE in ICH model, second, whether E2 ameliorated early HE and improved neurobehavioral performance, and third, investigated the mechanisms of E2 on BBB protection. We found out that hyperglycemia can enlarge HE and deteriorate neurological deficits as early as 3 hours after dextrose injection in ICH mice. Administration of E2 significantly decreased the hemorrhagic volume and improved the neurological deficits both 24 and 72 hours after ICH. E2 suppressed the activity of MMP-9 through ER\( \alpha \)/Sirt1/NF-\( \kappa \)B signaling pathway, and inhibition of ER\( \alpha \)/Sirt1 abolished the effects of E2 on the activity of MMP-9.
effect of E2. These observations indicated that E2 might serve as an alternative therapeutic strategy to prevent early HE in ICH patients by protecting BBB.

In clinic, hyperglycemia is more common in patients with preexisting diabetes mellitus, but is also present in a significant proportion of nondiabetic patients. About 60% ICH patients might develop hyperglycemia even in the absence of a previous history of diabetes mellitus, which is probably a response to the stress and severity of ICH. Hyperglycemia is strictly associated with greater HE and worse clinical outcomes after ICH. The deleterious effects of hyperglycemia are attributed to its secondary effects of acidosis, increased free radical formation, and release of inflammatory cytokines, which accelerated the degradation of BBB components and impaired the integrity of adjacent vessels surrounding the initial bleeding site. In streptozotocin-induced diabetic rats and mice, hyperglycemia results in greater HE after ICH. In the present study, we injected dextrose 3 hours after ICH to mimic hyperglycemia on hospital admission. We found that a greater macroscopic bleeding area was observed around the injection site, and more bleeding volume was confirmed in the hyperglycemic mice from 3 hours after dextrose injection when compared with saline controls. Our findings demonstrated for the first time that hyperglycemia in the acute setting of ICH significantly enhanced early HE and deteriorated neurological outcome. Liu et al in their excellent study demonstrated that hyperosmolarity caused by hyperglycemia led to a HE in autologous blood model of ICH via activation of kallikrein/platelet signaling pathway. Our findings show that 50% mannitol did not significantly affect the HE (Figure III in the online-only Data Supplement). HE in Liu’s publication was specified as hematoma area in the subarachnoid space. The hematoma volume was not measured by the authors. We, on the contrary, investigated effect of dextrose on the hematoma volume, without examination of the hematoma location. We think that, because of different scientific targets, there are no contradictions in these 2 studies.

Even though the precise mechanism of early HE during the acute phase of ICH is poorly understood, it is partly preventable. Inflammatory cascade activation and matrix metalloproteinases (MMPs) overexpression have been claimed to be the major perpetrators in BBB disruption and HE formation after ICH. Recently, emerging evidence from basic research suggests that estrogens showed potency and efficacy on BBB protection, which might contribute to preventing HE formation in ICH. After brain injury, estrogen exposure ameliorated BBB disruption induced by transient focal cerebral ischemia through inhibition of MMP-2 and MMP-9 activation. In female rats, endogenous estrogen reduced brain edema and improved neurological deficits after ICH when compared with male rats. In collagenase-induced ICH rats, estrogen treatment significantly reduced bleeding and lesion volume. In agreement with this, we observed activation of MMP-9 and HE in hyperglycemic ICH mice, and E2 dramatically suppressed the activity of MMP-9 and reduced early HE. These results justified that early HE is a potential therapeutic target in the acute phase of ICH, and E2 treatment may be an accessible and effective strategy to restrict HE and improve neurological functions in clinic.

Next we addressed the role of ERα in HE suppression of E2 after ICH. There are two receptor isoforms of E2, ERα, and ERβ; both of which are members of the nuclear receptor transcription factor superfamily. Deficiency of ERα but not of ERβ abolished the protective effect of E2 in ovariectomized mice subjected to focal cerebral ischemia. Additional studies confirmed that in animals subjected to SAH, there was a significant change in protein expression of ERα but not ERβ in dentate gyrus, and E2 reversed SAH downregulated ERα and phospho-Akt expression via an ERα-dependent mechanism. The results of these studies demonstrated that ERα, and not ERβ, was the critical responsible for estrogen-mediated neuroprotection in the rodent cerebral cortex. Furthermore, Vegeto et al revealed that ERα mediated anti-inflammatory activity of E2 in brain through inhibiting the expression of MMP-9. In transient cerebral ischemia, E2 has been proved attenuated BBB disruption by suppressing the activity of MMP-2 and MMP-9. In our experiment, we observed a decreased expression of ERα and activation of MMP-9 in hemorrhagic hemisphere, which is in agreement with the previous studies. Administration of E2 and ERα inhibitor MPP did not affect the level of ERα, whereas MPP abolished the beneficial effects of E2 on BBB broken. Our data demonstrated that the protective role of E2 on BBB is partially mediated by inhibiting the activity of MMP-9 through ERα in ICH rats.

How does E2/ERα signaling regulate MMP-9? ERα is a member of the nuclear receptors, and once ERα is activated, ERα may interact with specific transcriptional coregulators to modulate gene expression and mediate the effects of E2. The role of sirtuins in the regulation of ER signaling has emerged over the years. Sirtuins are a family of highly conserved protein deacetylases that have extensively implicated energy metabolism, stress response, and cell/tissue survival. Sirt1 is the best characterized family member, resides mainly in the nucleus and deacetylates numerous transcription factors, including p53, NF-κB, forkhead box O, and so on. Exciting researches have shown that in a rat model of postmenopausal metabolic syndrome, E2 restored the protein expression of Sirt1 and alleviated endothelial dysfunction. E2 treatment also has been reported to result in an upregulation of Sirt1 in old mice and in Hela cells. The studies of Elangovan showed that ERα bound to p53 promoter and suppressed its expression and that the process is obligatorily dependent on Sirt1 in breast cancer cells. In the present study, E2 treatment promoted deacetylation of NF-κB and inactivated its transcription of MMP-9. Sirt1 knockdown abolished this effect, indicating that Sirt1 is necessary for the ability of E2/ERα signaling to suppress MMP-9 and attenuate HE in hyperglycemic ICH rats.

In conclusion, we demonstrated that hyperglycemia enhanced early HE in acute setting of ICH, and administration of E2 prevented the development of HE and improved neurological deficits in ICH mice. E2 preserved the integrity of BBB by suppressing the activity of MMP-9, which is dependent on ERα/Sirt1/NF-κB pathway. Our results suggested that E2 might be a promising approach to restrict early HE after ICH.
**Disclosures**

None.

**References**

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Supplementary figures

A. Experiment 1

Experimental design.

dx or saline was injected intraperitoneally 3 hours after ICH. Blood glucose was measured at 3h, 4h, 5h and 6h after ICH. Hemorrhagic volume and Garcia scores were detected at 6h, 24h, and 72h after ICH. Experiment 2 is to study the outcome of E2 on hyperglycemia enhanced HE and neurological deficits in ICH mice. E2 was administrated intraperitoneally at 1h after ICH. Hemorrhagic volume and Garcia scores were detected at 24h and 72h after ICH.

B. Experiment 2

Experimental design.

Collagenase DX/Saline

0h 3h 4h 5h 6h 24h 72h

C. Experiment 3

Sirt1 siRNA Collagenase MPP E2 DX

-48h 0h 45min 1h 3h 24h

Hemorrhagic volume Garcia scores Western blot: ERα, Sirt1, NF-κB

Zymography: MMP-9

Supplementary figure I. Experimental design. Experiment 1 is to investigate the effects and time course of hyperglycemia on HE (A). DX or saline was injected intraperitoneally 3 hours after ICH. Blood glucose was measured at 3h, 4h, 5h and 6h after ICH. Hemorrhagic volume and Garcia scores were detected at 6h, 24h, and 72h after ICH. Experiment 2 is to study the outcome of E2 on hyperglycemia enhanced HE and neurological deficits in ICH mice. E2 was administrated intraperitoneally at 1h after ICH. Hemorrhagic volume and Garcia scores were detected at 24h and 72h after ICH. C, Experiment 3 is to investigate the potential mechanisms of E2 on BBB protection (C). ERα inhibitor MPP or Sirt1 siRNA was administrated at 45min after or 48h before ICH respectively; hemorrhagic volume and Garcia scores were detected at 24h after ICH. Protein expression of ERα, Sirt1, acetylated NF-κB, and activity of MMP-9 were detected.
Supplement figure II. Representative western blots 48 hours after administration of Sirt1 siRNA in Sham and ICH animals. Sirt1 siRNA strongly knocked down the expression of Sirt1 in both Sham and ICH mice compared with scramble siRNA. *p<0.05 vs. Sham+Scramble siRNA; # p<0.05 vs. ICH+DX+Scramble siRNA. n=6 for each group.
Supplement figure III. Hemorrhagic volume and neurobehavioral scores with or without mannitol administration 24 hours after ICH. Mannitol had no effects on Hemorrhagic volume (A) and neurobehavioral scores (B) when injection at 3 hours after ICH compared with ICH+Vehicle group. *p<0.05 vs. Sham. Sham, n=6; ICH+Vehicle, n=5; ICH+Mannitol, n=5.