2-Methoxyestradiol Inhibits Intracerebral Hemorrhage-Induced Angiogenesis in Rats

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ABSTRACT

AIM: Angiogenesis occurs after intracerebral hemorrhage (ICH). Hypoxia-inducible factor-1α (HIF-1α) is a critical regulator of angiogenesis. However, its role in the central nervous system remains controversial. 2-Methoxyestradiol (2ME2), a natural metabolite of estrogen, is known to inhibit HIF-1α. In the present study, we investigated the effect of 2ME2 in a rat model of ICH-induced angiogenesis.

MATERIAL and METHODS: Sprague-Dawley male rats (n=50) were randomly divided into 5 groups: Sham operated group; ICH; ICH+2ME2; and ICH+Vehicle groups. ICH model was induced by stereotactic injection of collagenase type VII into the right globus pallidus. 2ME2 or vehicle (10% dimethyl sulfoxide) was administered intraperitoneally 10 min after ICH. Angiogenesis and expression of HIF-1α was evaluated by immunohistochemistry, quantitative real time-reverse transcription polymerase chain reaction and western blot, respectively.

RESULTS: Proliferating cell nuclear antigen (PCNA)-labeled nuclei were detected in cerebral endothelial cells (ECs) around the hematoma. The labeling peaked at 14 days post-ICH. HIF-1α-immunoreactive microvessels with dilated outline were detected in the perihematomatol tissues. The vessels extended into the clot from the surrounding tissues from day 7 onwards. HIF-1α protein levels increased, while no change was observed in HIF-1α mRNA expression after ICH. 2ME2 decreased the PCNA-labeled nuclei in cerebral ECs and down-regulated the expression of HIF-1α protein as well, while it had little effect on the mRNA expression of HIF-1α.

CONCLUSION: HIF-1α inhibitor, 2ME2, inhibited post-ICH angiogenesis by suppressing HIF-1α expression, thus exerting detrimental effects in ICH.

KEYWORDS: 2-methoxyestradiol, Intracerebral hemorrhage, Angiogenesis, Hypoxia-inducible factor-1α

INTRODUCTION

Intracerebral hemorrhage (ICH) is a life-threatening catastrophe caused by bleeding in the brain parenchyma. Although it accounts for only about 15 to 20% of all stroke events, ICH has higher mortality and disability rates as compared to other types of stroke (7,21). Despite outstanding progress in the treatment of hemorrhagic stroke in the past 10 years, little progress has been achieved in ICH treatment.

Angiogenesis, the formation of capillaries from preexisting vessels, plays a central role in a variety of physiological and pathological conditions (8). During angiogenesis, endothelial cells stimulated by angiogenic factors start to proliferate.
Hence, cell proliferation markers such as proliferating cell nuclear antigen (PCNA), and endothelial cell marker, von Willebrand factor (vWF), are frequently used in angiogenesis studies (15). The molecular events governing angiogenesis are complex and involve multiple families of proteins and receptors. Hypoxia-inducible factor-1α (HIF-1α) is one of the major transcriptional activators of a set of angiogenic genes such as vascular endothelial growth factor (VEGF) (28). In our previous studies, we demonstrated ICH-induced angiogenesis in rat brains, which was accompanied by up-regulation of HIF-1α (12,32).

2-Methoxyestradiol (2ME2) is an endogenous metabolite of estrogen that is known to inhibit HIF-1α in a dose-dependent manner (17). Administration of 2-ME2 has been shown to inhibit angiogenesis in both in vivo and in vitro manner (17). Administration of 2-ME2 has been shown to inhibit angiogenesis in both in vivo and in vitro studies (19,34). The present study was designed to clarify the effect of 2ME2 on ICH-related angiogenesis.

# MATERIAL and METHODS

## Animal Preparation

Adult male Sprague–Dawley (SD) rats (weight: 250-300 g each) were housed under diurnal lighting conditions. This study was carried out in strict compliance to the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (NIH Publication No. 85-23, revised 1996). All experiments were approved by the Institutional Animal Care and Use Committee of China Three Gorges University.

## Induction of Intracerebral Hemorrhage

ICH was induced by collagenase according to the previous protocol (18). After anesthesia with chloral hydrate (400 mg/kg) via intraperitoneal injection, the animals were fixed in a prone position on a stereotactic frame (STOELTING Co., USA). Following a scalp incision, a small cranial burr was drilled near the right coronal suture, 3.2 mm lateral to the midline. Bacterial type VII collagenase (0.5 U in 2.5 μL 0.9% sterile saline, Sigma Co., USA) was slowly injected into right globus pallidus (1.4 mm posterior and 3.2 mm lateral to bregma, 5.6 mm ventral to the cortical surface) with a 5-μL Hamilton syringe for over 5 minutes. The needle was left there for another 5 minutes. The burr hole was sealed with bone wax, and the wound sutured. The animals were placed in a warm box to recover individually. In the sham group, the rats were injected with 2.5 μL 0.9% sterile saline without collagenase at the same site. During the procedure, rectal temperature was monitored and maintained at 37.5°C using a feedback controlled heating pad.

## Drug Administration

2ME2 (Sigma-Aldrich Corp, MO), an HIF-1α inhibitor, was dissolved in PBS with 10% dimethyl sulfoxide (DMSO). 2ME2 (15 mg/kg, intraperitoneal injection) was administered to rats 10 minutes after induction of ICH (34). Rats in the DMSO treated group were administered the same volume of vehicle (DMSO diluted in PBS) at the same time point after induction of ICH. No treatment was administered to the ICH alone and sham-operated animals.

## Neurological Evaluation

The forelimb asymmetry test was performed as described by Hua et al.(5). All rats were laid in a transparent cylinder, 20 cm in diameter and 30 cm in height. A mirror was placed near the cylinder at an angle convenient for observing the forelimb movements of the rat; and at the same time, simultaneous recording was performed by a video camera. The test lasted 10 minutes. Rat behavior was quantified by recording the number of occasions when the rat’s forelimb touched the cylinder wall while it was in an orthostatic position with a balanced centre of gravity. The occasions of contact with the unimpaired (ipsilateral) forelimb were recorded as I, that with the impaired forelimb (contralateral to the collagenase injection site) as C, and movement of both forelimbs was recorded as B. The forelimb asymmetric use rate (AUR) was calculated using the following formula:

\[
AUR = \left[ \frac{I}{I+C+B} \right] - \left[ \frac{C}{I+C+B} \right]
\]

The result was evaluated by a researcher who was blinded to the experimental design.

## Specimen Preparation

Randomly chosen animals from the groups were deeply anesthetized with chloral hydrate (800 mg/kg). For immunohistochemistry, animals (n=5 per time point) were transcardially perfused with 0.9% saline followed by 250 mL ice-cold 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH=7.4). The excised brains were post-fixed in the same fixative for 2 hours, and subsequently transferred to 20% and then 30% sucrose in 0.1 M PB (pH=7.4) sequentially at 4°C until sinking. Coronal sections of brain (30-μm thick) were prepared at -20°C with a cryostat (CM1900, Leica, Germany), some of which were collected in 0.01 M phosphate-buffered saline (pH=7.4) and stored at 4°C. For reverse transcription-polymerase chain reaction (RT-PCR) and western blot, rats (n=10 per time point) were transcardially perfused with 0.9% saline. The brains were immediately removed, and the tissues in striatum adjacent to the hematoma were dissected and frozen at -196°C in liquid nitrogen.

## Immunohistochemistry

Under deep anesthesia with chloral hydrate (800 mg/kg), the collagenase-induced ICH animals (n=5, per time point) were randomly chosen at days 3, 7, and 14 post-operation, and were transcardially perfused with 0.9% saline followed by 250 mL ice-cold 4% paraformaldehyde in 0.1 M phosphate buffer (pH=7.4). The brains were removed and post-fixed in the same fixative for 2 hours, then transferred to 20% and 30% sucrose in 0.1 M phosphate buffer (pH=7.4) sequentially, at 4°C until sinking. Coronal sections of brain (30 μm) were prepared at -20 °C with a cryostat (CM1900, Leica Co., Germany) for immunohistochemical staining.

## Proliferation of Cerebral Microvascular Endothelial Cells

Sections were pretreated to denature DNA as described below. After being immersed in 50% formamide /2× saline-sodium citrate buffer (SSC, pH=7) at 65°C for 2 hours, sections were washed with 2× SSC for 10 minutes, followed by their incubation in 2 N HCl at 37°C for 30 minutes. The nonspecific antigen was blocked by 5% bovine serum albumin (BSA,
Sigma, USA) and sections were incubated overnight with a mouse anti-PCNA (1:250, Santa Cruz Biotech, USA) at 4°C. The biotinylated anti-mouse IgG (1:200, Vector Laboratories, USA) was used for 1 hour at 37°C, and then treated with avidin-biotin-peroxidase complex (ABC) (1:100, Vector Laboratories, USA) at 37°C for 1 hour. Immunoreactivity was assessed with 3,3'-diaminobenzidine (DAB, Boster Biotech, China).

To detect the proliferated cerebral microvascular ECs, we adopted the double immunolabeling method. The procedure in the above protocol was followed after DNA denaturation, but the primary antibody was changed to rabbit anti-vWF (1:400, Chemicon International, USA), which was used to label cerebral ECs. The visualization was enhanced with ammonium nickel sulfate. The slides were observed at 40x objective magnification and the nuclei with PCNA+/vWF- close to the hematoma were counted (four 250×250 μm areas) in randomly selected 5 sections for each animal. The data were analyzed by Motic Images Advance 3.2 image analysis software, and the results are presented as number of nuclei per mm² (N/mm²). The result was evaluated by a researcher who was blinded to the experimental design.

**Spatial Profile of HIF-1α after ICH**

Immunohistochemical examination was performed to observe spatial profiles of HIF-1α. Briefly, sections were brought to room temperature and incubated in 3% H₂O₂ for 15 minutes. After washing 3 times in phosphate-buffered saline for 5 minutes each, nonspecific binding was blocked in 5% bovine serum albumin (Sigma Aldrich Co.) at 37°C for 1 hour. Sections were incubated with mouse anti-HIF-1α (1:200, Santa Cruz Biotech), with a biotinylated anti-mouse immunoglobulin G (1:200) for 1 hour, and then with avidin-biotin-peroxidase complex (1:100, Vector Laboratories) at 37°C for 1 hour. Immunoreactivity was visualized with diaminobenzidine (DAB, Boster Biotech Co.).

Immunofluorescence double labeling was used to detect the expression of HIF-1α in ECs. The sections were first incubated for 48 hours at 4°C with a mixture of two primary antibodies against vWF (1:200) and HIF-1α (1:100). The following secondary antibodies were used: Fluorescein isothiocyanate-conjugated donkey anti-rabbit antibody (1:50, Santa Cruz Biotechnology) for vWF detection; rhodamine-conjugated donkey anti-mouse antibody (1:100, Santa Cruz Biotechnology) for HIF-1α. These sections were scanned using a laser scanning confocal microscope (LSM-510, Zeiss, Germany).

For the negative control, 1% BSA was used instead of the primary antibody in each experiment.

**Quantitative Real-Time RT-PCR**

Total RNA was purified from 100 mg tissue near the hematoma in all groups using TRIzol reagent (Invitrogen, Carlsbad, California). The integrity of total RNA was assessed on agarose gel electrophoresis; the purity and concentration were detected by a spectrophotometer (UV-1201, Shimadzu). Reverse transcription was performed on 2 μg of total RNA using 1 μg/μL oligo (dT) 18 (1 μL), 10 mM dNTP Mix (2 μL), RNase inhibitor (1 μL) and 200 U/μL M-Mulv-Reverse Transcriptase (1 μL) at 70°C for 5 minutes, at 37°C for 5 minutes, at 42°C for 60 minutes and 70°C for 10 minutes following the manufacturer’s instructions (Fermentas, CA, USA). cDNA was stored at -20°C. PCR amplification was performed using SYBR Premix ExTaq™ PCR kit (4 μL of 1:2 cDNA dilution was used, Takara Biotechnology, Japan) in a LightCycler Real-Time Detection System (Roche Diagnostics Limited, Germany). The following thermocycling protocol was used: 10 seconds at 95°C; 30 cycles of 5 seconds at 95°C, 20 seconds at 52°C , and 10 seconds at 72°C; and a melting curve at 60°C. Primers for HIF-1α and β-actin were designed with Primer Premier 5.0 software for rat (PRIMER Biosoft International, CA, USA) as follows:

HIF-1α, sense 5 were designed with Primer Premier 5.0 software for rat (PRIMER Biosoft Inter β-actin, sense 5'-CGTTGACATCCGTAAGAC -3’ and antisense 5'-TGGAAAGTGGACAGTGG -3'.

Melting curves of all samples were prepared as controls to test specificity. All gene expression data were calculated by 2−ΔΔCT, which indicates an n-fold change in gene expression relative to the sham control sample (11).

**Western Blot**

Protein was extracted from the striatal tissues adjacent to the hematoma. Western blot analysis was performed (23). Briefly, 50-μg proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to a Hybond-C pure nitrocellulose membrane (Amersham). Monoclonal mouse antibodies for HIF-1α (1:100) and β-actin (1:200, Santa Cruz Biotech, CA, USA) were used. Quantitative densitometric analysis of Western blots was performed with a computerized digital image analysis system.

**Statistical Analysis**

All data are expressed as mean ± standard deviation (SD). One-way Analysis of Variance (ANOVA) was used to compare between-group the differences. P value less than 0.05 was considered statistically significant.

**RESULTS**

**Neurological Evaluation**

The AUR for all the groups are shown in Figure 1A, B. No neurological deficit was observed in the sham operated rats. After ICH, the rats were observed for neurological deficit: the AUR gradually decreased as the observation time progressed (Figure 1A, p<0.01). After 2ME2 treatment, the AUR values notably increased from 7 days as compared to those in the ICH+DMSO groups (Figure 1B, p<0.05).

**Angiogenesis Following ICH**

After ICH, some PCNA-positive cells in von Willebrand factor (vWF) - immunoreactive dilated vessels were detected around the clot (Figure 2A, B), and the PCNA+/vWF+ nuclei increased notably over time (Figure 2C, p<0.05). After 2ME2 treatment, the number of PCNA+/vWF+ nuclei was decreased significantly.
as compared to that in the ICH+DMSO groups (Figure 2D, p<0.05).

**Spatial Distribution of HIF-1α after ICH**

The immunohistochemical staining of HIF-1α is shown in Figure 3. In the sham-operated animals, few HIF-1α-positive vessels were observed (Figure 3A). However, some HIF-1α-positive dilated vessels were detected mainly in the perihematomal tissue, and these positive vessels extended into the clot from 7 days after ICH (Figure 3B). Furthermore, immunofluorescent double labeling confirmed that HIF-1α was localized to vWF+ vessels around the hematoma (Figure 3C-E).

**HIF-1α mRNA and Protein Analysis**

No signals of HIF-1α mRNA and protein were detected in the sham-operated rats. After ICH induction, the HIF-1α protein levels increased markedly at 3 days, and then declined. However, HIF-1α mRNA levels were unchanged. The levels of HIF-1 protein in the 2ME2+ICH group were significantly lower than those in the ICH+DMSO groups (p<0.05). However, 2ME2 had little effect on the expression of HIF-1α mRNA (Figure 4A, B).

![Figure 1: Neurological evaluation. Behavioral tests were implemented in rats after ICH or sham operation. The ICH group was distinctly worse impaired as compared to the sham group at the corresponding time points. The AUR values of the ICH+2ME2 group were significantly higher than that of ICH+DMSO group from 7 days post ICH. (*p<0.05, **p<0.01, n=10).](image)

![Figure 2: Proliferated cerebral endothelial cells after ICH. Post-ICH induction, some PCNA-positive cells (brown, arrow) in vWF-immunoreactive dilated vessels (blue) were detected around the hematoma (A, B); the PCNA+/vWF+ nuclei increased up till 14 days (C); 2ME2 markedly increased the expression levels of PCNA-positive cells in the vWF+ immunoreactive vessels (D). * p<0.05, ** P<0.01. Scale bar, 50 μm for panel A, 25 μm for panel B.](image)
Figure 3: Immunohistochemical examination for detection of HIF-1α. HIF-1α positive signals were hardly observed in the brains of sham-operated animals (A). After ICH, HIF-1α immunoreactive microvessels with the dilated outline were detected in the perihematomal tissues (B). Immunofluorescent double labeling showed that HIF-1α (red) was localized in vWF⁺ (green) vessels after ICH (C-E). Scale bar, 50 μm for panels A-B, 100 μm for panels C-E.

Figure 4: Quantitative analysis of HIF-1α mRNA and protein after ICH. After induction of ICH, HIF-1α mRNA levels did not change at any time-point in all groups (A), while the HIF-1α protein level increased at 3 days followed by a decline. 2ME2 strikingly down-regulated the HIF-1α protein expression (B). *p<0.05, **p<0.01.
DISCUSSION

In the present study, we demonstrated detrimental effects of 2ME against hemorrhagic injury in rats. After ICH, PCNA-positive cells were detected in the vWF+ immunoreactive dilated vessels around the clot. Further, a notable increase in the PCNA+/vWF+ nuclei was observed accompanied by enhanced expression of HIF-1α. 2ME2 decreased the number of PCNA+/vWF+ nuclei by down-regulating the expression of HIF-1α. The findings indicate that HIF-1α plays a critical role in ICH-induced angiogenesis, that 2ME2 is a potent inhibitor of angiogenesis.

HIF-1α is a helix-loop-helix transcription factor that is ubiquitously expressed in mammalian cells. Under normoxic conditions, HIF-1α is rapidly degraded by proteasome (6,22). Hence, no HIF-1α signal could be detected in the sham control group. Other studies have also demonstrated a central role of HIF-1α in angiogenesis (25,33). In our previous study, we found abundant HIF-1α immunoreactive microvessels in ECs in the vicinity of the clot (12,32). The spatiotemporal profile is also consistent with post-ICH angiogenesis. Therefore, it is tempting to suppose that HIF-1α may be a crucial mediator for ICH-induced angiogenesis.

To confirm this presumption, 2ME2, a HIF-1α inhibitor, was used in this study. 2ME2, a natural metabolite of estradiol, has been shown to inhibit tumor growth and angiogenesis by inducing dysregulation of HIF-1α (13,14). At a dose of 16 mg/kg, 2ME2 can effectively inhibit the expression of HIF-1α (31,34). 2ME2 not only depolymerizes microtubules and blocks nuclear accumulation of HIF-1α and HIF-transcriptional activity, but also downregulates HIF-1α at the posttranscriptional level (1,14). Consistent with other studies, the present study showed that 2ME2 could inhibit ICH-induced angiogenesis by downregulating HIF-1α at protein levels and without altering HIF-1α transcriptional level.

The role of HIF-1α in central nervous system remains controversial. Inhibition of HIF-1α was shown to have a neuroprotective effect after cerebral ischemia, which was associated with preservation of blood brain barrier integrity (9,30), amelioration of brain edema (3,16), and prevention of neuronal death (2). Moreover, Yan et al. reported that 2-ME2 reduced cerebral vasospasm after subarachnoid hemorrhage by inhibiting HIF-1α (24). Additionally, normobaric oxygen therapy was shown to reduce neurological deficits by suppressing HIF-1α expression in the perihematoma (26). However, some conflicting findings have also been reported on the exact role of HIF-1α after cerebral ischemia (19,20,29,31) and ICH (10,27). In the present study, inhibition of HIF-1α by 2ME2 not only reduced post-ICH angiogenesis but also delayed functional recovery. The discrepancy in findings may be attributable to different time-frame, different stroke models or different inhibitors of HIF-1α used in these studies. Intriguingly, inhibition of HIF-1α by 2ME2 aggravated ICH-induced neurological deficits from 7 days, which indicated that 2ME2 exerted detrimental effects at the late stage of ICH. Since HIF-1α is also involved in neurogenesis after stroke (4), the effects of 2ME2 on ICH-related neurogenesis requires further investigation.

CONCLUSION

We demonstrate for the first time that the HIF-1α inhibitor, 2ME2 impaired post-ICH angiogenesis and functional recovery. These findings indicate that HIF-1α may have a protective effect against ICH-induced damage. However, further investigations are essential to elucidate the precise mechanisms of HIF-1α action before proposing it as a neuroprotective target for the treatment of ICH.

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