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Research Paper

# Pretreatment with low-dose fimasartan ameliorates NLRP3 inflammasome-mediated neuroinflammation and brain injury after intracerebral hemorrhage

Xiuli Yang[a,b,c], Jing Sun[b,d], Tae Jung Kim[a,b], Young-Ju Kim[a,b], Sang-Bae Ko[a,b], Chi Kyung Kim[e], Xiaofeng Jia[c,f,g,h,i,**], Byung-Woo Yoon[a,b,j,*]

[a] Department of Neurology, Seoul National University Hospital, Seoul 03080, Republic of Korea
[b] Biomedical Research Institute, Seoul National University Hospital, Seoul 03080, Republic of Korea
[c] Department of Neurosurgery, University of Maryland School of Medicine, Baltimore, MD 21201, USA
[d] Department of Neurology, China-Japan Union Hospital of Jilin University, Changchun, 130000 Jilin, China
[e] Department of Neurology, Korea University Guro Hospital, Seoul 08308, Republic of Korea
[f] Department of Orthopaedics, University of Maryland School of Medicine, Baltimore, MD 21201, USA
[g] Department of Anatomy Neurobiology, University of Maryland School of Medicine, Baltimore, MD 21201, USA
[h] Department of Biomedical Engineering, The Johns Hopkins University School of Medicine, Baltimore, MD 21205, USA
[i] Department of Anesthesiology and Critical Care Medicine, The Johns Hopkins University School of Medicine, Baltimore, MD 21205, USA
[j] Neuroscience Research Institute, Seoul National University College of Medicine, Seoul 03087, Republic of Korea

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ABSTRACT

Nucleotide-binding and oligomerization domain-like receptor family pyrin domain-containing 3 (NLRP3) inflammasome, which is composed of an NLRP3 domain, the adaptor molecule apoptosis-associated speck-like protein containing a CARD (ASC) domain, and procaspase-1, plays an important role in the immune pathophysiology of the secondary damage induced by intracerebral hemorrhage (ICH). This study aims to investigate whether pre-stroke treatment with fimasartan, an angiotensin II receptor blocker, has anti-inflammatory effects on ICH by inhibiting the activation of the NLRP3 inflammasome. Sprague-Dawley rats were divided into five groups: sham, vehicle, low-dose (0.5 mg/kg) and regular-doses (1.0 and 3.0 mg/kg) fimasartan. These rats were treated for 30 days before the induction of collagenase-induced ICH and continuously 3 days after surgery. The mean blood pressure (BP) in the low-dose fimasartan group was not significantly different from that of control, and BP in the regular-dose groups was decreased in a dose-dependent manner. Pretreatment with low-dose fimasartan attenuated ICH-induced edema and improved neurological functions. Activation of the NLRP3/ASC/caspase-1 and the NF-κB pathways after ICH was markedly reduced by low-dose fimasartan. The double immunofluorescence staining of brain cells showed a significant decrease in the co-localization of NLRP3 with Iba1 (microglia marker) positive cells by fimasartan treatment. Cultured microglia cells stimulated by hemolysate demonstrated significant activation of the inflammasome, which was reduced by fimasartan. Pretreatment with a low-dose fimasartan alleviated brain damage after acute ICH by inhibiting the NLRP3 inflammasome without lowering MBP. Our study suggests pre-stroke administration of fimasartan could potentially attenuate ICH-induced secondary brain injury by targeting the inflammasome.

## 1. Introduction

Intracerebral hemorrhage (ICH) is one of the subtypes of stroke that is characterized by high mortality and morbidity, and represents approximately 10–15% of strokes worldwide each year (Keep et al., n.d.; Lan et al., 2017). The number of hospital admissions for ICH has increased in the past ten years. Despite the recent advance in stroke therapy, the mortality rate has not decreased due to the increased elderly population, the racial differences in the incidence of ICH, and the increased use of anticoagulants (Qureshi et al., n.d.; Qureshi et al.,

* Correspondence to: Byung-Woo Yoon, Department of Neurology, Seoul National University Hospital, Seoul 03080, Republic of Korea.
** Correspondence to: Xiaofeng Jia, Department of Neurosurgery, University of Maryland School of Medicine, 10 South Pine Street, MSTF 559, Baltimore, MD 21201, USA.
*E-mail addresses:* xjia@som.umaryland.edu (X. Jia), bwyoon@snu.ac.kr (B.-W. Yoon).

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2001). Currently, there are no effective therapies for ICH because of the complex and poorly understood mechanisms. Accumulating evidence suggests that inflammatory factors are involved in the secondary brain damage induced by ICH (Yang et al., 2017); however, the molecular mechanisms underlying the innate immune response in ICH are not fully understood (Iadecola and Anrather, 2011; Wang and Doré, 2007).

Recently, the so-called inflammasome has been found to regulate diverse inflammatory responses in all organs, including the brain (Strowig et al., 2012). The inflammasome plays a key role in the innate immune response in the central nervous system (CNS) diseases (Heneka et al., 2017). Typically, the inflammasome is composed of at least one member of the cytosolic innate immune sensor family, the NOD-like receptors (NLRs), coupled with the adaptor apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC), and caspase-1 (Schroder and Tschopp, 2010). The nucleotide binding and oligomerization domain-like receptor family pyrin domain-containing 3 (NLRP3) inflammasome is the best characterized and the most clinically relevant inflammasome to date. Activation of the NLRP3 inflammasome subsequently activates caspase-1 and induces the secretion of mature cytokines, such as interleukin-1β (IL-1β) (Zhu et al., 2017). The processing of mature cytokines is mediated by two distinct signals. The first signal is the priming signal, which involves activation of the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) signaling pathway. The second signal involves the formation of the NLRP3 inflammasome complex with cleaved caspase-1 (Franchi et al., 2012). An increasing number of studies have suggested that the NLRP3 inflammasome contributes to brain inflammation in stroke, especially in ICH (Fann et al., 2013; Ma et al., 2014).

It is well-known that chronic hypertension is by far the most common risk of bleeding that can lead to ICH and that angiotensin II receptor antagonists (ARBs) are one of the most effective management options for hypertension (Saavedra, 2012). ARBs collectively have neuroprotective effects, although the underlying mechanisms of action may differ. Previous studies found that patients treated with ARBs or angiotensin-converting enzyme inhibitors (ACEI) prior to ischemic stroke onset are associated with lower stroke severity and better outcome, which may be indirectly related to effect due to the lowered BP (Fuentes et al., 2010; Selim et al., 2005). These drugs may have a potential neuroprotective effect and lower the stroke risk for stroke patients. A recent study found pre-stroke ARB treatments might reduce the 30-day mortality of ischemic stroke patients (Sundboll et al., 2015). Fimasartan (BR-A-657), a new ARB drug, has a stronger affinity for the AT1 receptor subtype and presents superior inhibitory activity compared to other ARBs (Chi et al., 2011a). Pre-stroke use of fimasartan has been found to be protective in ischemic stroke and myocardial infarction (Han et al., 2013; Kim et al., 2015). However, the effect of pretreatment of fimasartan on ICH has remained unknown.

Therefore, this study aims to evaluate the dose-dependent effect of fimasartan administration in acute ICH stroke and investigate the mechanism of neuroinflammatory attenuation that is beyond BP control. We conducted a 30-day pretreatment with different doses of fimasartan and validated its ability to regulate BP prior to ICH. We decided to move forward with the low-dose fimasartan as its effects on BP were statistically indistinguishable from controls, thus allowing us to isolate BP-independent mechanisms. Specifically, we investigated the role of low-dose fimasartan on two inflammatory signal pathways induced by ICH: the NLRP3 inflammasome and the NF-κB pathways.

## 2. Materials and methods

### 2.1. Fimasartan administration

Fimasartan (Boryung Pharmaceutical Company, Republic of Korea), was dissolved in phosphate-buffered saline (1 mg/mL) and diluted with sterile water to constitute either the low dose (0.5 mg/kg, p.o.) or regular doses (1.0 and 3.0 mg/kg, p.o.) according to our previous study (Kim et al., 2015). Fimasartan or distilled water (DW) was administered orally for 30 days prior to the induction of ICH, and continuously for 3 days after the surgery at the same time every morning.

### 2.2. Induction of intracerebral hemorrhage

All animal experimental protocols were performed in accordance with the relevant guidelines and regulations approved by the National Institutes of Health Animal Care and Use Committee of the Biomedical Research Institute at Seoul National University Hospital. Total 113 four-week-old male Sprague-Dawley rats (Koatech, Seoul, Republic of Korea) weighing 65–75 g were randomly separated into five groups: sham, ICH + DW, ICH + low-dose fimasartan (0.5 mg/kg, p.o.) and ICH + regular-doses fimasartan (1.0 and 3.0 mg/kg, p.o.). After one month, we performed the ICH surgery on adult rats at 8 weeks of age (250 - 300 g) as previously published (Song et al., 2003). The rats were then injected intrastriatally (3.0 mm left, 0.2 mm posterior to the bregma and 6.0 mm in depth) with collagenase IV (0.6 U in 1 μL saline, Sigma) in a stereotaxic frame (David Kopf Instruments, Tujunga, CA, USA). The forelimb flexion and contralateral circling were observed to confirm the success of the ICH surgery. Sham-operated rats only underwent needle insertion without collagenase injection. Male rats were used to avoid the neuroprotective effects of estrogens which may affect stroke outcome (Dubal and Wise, 2002).

### 2.3. Monitoring of blood pressure

Mean BP levels were noninvasively monitored at the same time from the rat's tail (homologated by Bland Altman Testing) (Ciocoiu et al., 2013) with a CODA Noninvasive Blood Pressure System (Kent Scientific Corporation, Torrington, CT) throughout the whole experiment including days 28, 21, 14, 7 and 3 prior to the surgery and at baseline with the administration of fimasartan ($n$ = 10 per group) (Fig. 1A).

### 2.4. Measuring the brain water content and hemorrhage volume

We analyzed the brain water content and hematoma volume 3 days after surgery because edema is the most severe three days after ICH (Xi et al., 2006). Rats were euthanized 72 h after ICH. The brains ($n$ = 12 per group) were then divided into two hemispheres along the midline and immediately weighed using an electric analytical balance to obtain the wet weight. The brain samples were then dried in a gravity oven at 100 °C for 24 h to obtain the dry weight. Water content = (wet content-dry content)/(wet content) × 100% (Song et al., 2003).

To evaluate the hemorrhagic lesion volume, the brains ($n$ = 8 per group) were serially sectioned at 1-mm intervals in the coronal plane through the needle entry site. The hematoma area of each section was assessed by Image J (National Institutes of Health, Bethesda, MD). The total hematoma volume ($mm^3$) was calculated by summing the hematoma area in each section and multiplying by the thickness of the sections (Kim et al., 2013).

### 2.5. Behavioral testing

Two behavioral tests, the modified Neurological Severity Score (mNSS) and forelimb placing test, were performed by investigators blinded to the groups. The rats were assessed before and 1 h, 1 day and 3 days after ICH ($n$ = 17 per group). The mNSS test includes a composite of the motor (six points), sensory (two points), and beam balance (six points) tests, in addition to the absence of reflexes and presence of abnormal movements (four points). The mNSS is graded on a scale of 0–18 (normal score = 0; maximal deficit score = 18) to determine impairment (Chen et al., 2001).

The vibrissae-elicited forelimb placing test was used to assess the asymmetry between forelimbs (Schallert et al., 2000). The rats were held to allow their forelimbs hanging free. Each forelimb was tested by