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PEROXYNITRITE DECOMPOSITION CATALYST PREVENTS MATRIX METALLOPROTEINASE-9 ACTIVATION AND NEUROVASCULAR INJURY AFTER HEMOGLOBIN INJECTION INTO THE CAUDATE NUCLEUS OF RATS

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Abstract—Hemoglobin (Hb) is a major constituent of blood and a potent mediator of oxidative or nitrative stress after intracerebral hemorrhage (ICH). Our previous study demonstrated that Hb could induce abundant peroxynitrite ($ONOO^-$) formation *in vivo*, which may be involved in the blood–brain barrier (BBB) disruption, however, the drug intervention is absent and also the underlying mechanism. Using an experimental stroke model by injecting Hb into the caudate nucleus of male Sprague–Dawley rats, we assessed the role of $ONOO^-$ decomposition catalyst, 5,10,15,20-tetrakis (4-sulfonatophenyl) porphyrinato iron(III) [FeTPPS] in the activation of MMP-9 and Hb-induced neurovascular injuries. 3-Nitrotyrosine (3-NT, as an index of $ONOO^-$ formation) and NF-κB expression was measured by western blot (WB) and immunohistochemistry (IHC)/immunofluorescence (IF). Activity of MMP was evaluated by *in situ* zymography. Neurovascular injury was assessed using zonula occludens-1 (ZO-1) by WB and IF, fibronectin (FN) and neuron-specific nuclear protein (NeuN) IHC. Perihematomal cell death was determined by TUNEL assay. Behavioral outcome was measured by modified neurological severity score (mNSS) test. At the injured striata, profuse 3-NT was produced and mainly expressed in neutrophils and microglia/macrophages. 3-NT formation significantly colocalized with nuclear factor-κB (NF-κB) expression. *In situ* zymography showed that gelatinase activity was mostly co-localized with neurons and blood vessel walls and partly with neutrophils and microglia/macrophages. Enhanced 3-NT production, NF-κB induction and MMP-9 activation were obviously reduced after FeTPPS treatment. Hb-induced injury to tight junction protein (ZO-1), basal lamina of FN-immunopositive microvasculature and neural cells was evidently ameliorated by FeTPPS. In addition, apoptotic cell numbers as well as behavioral deficits were also improved. The present study shows that the administration of the $ONOO^-$ decomposition catalyst FeTPPS protects against Hb-induced neurovascular injuries and improves neurological function, which possibly in part by suppressing MMP-9 activation.

Key words: peroxynitrite, matrix metalloproteinases, neurovascular injury, hemoglobin, intracerebral hemorrhage, oxidative or nitrative stress.

INTRODUCTION

Intracerebral hemorrhage (ICH) is one of the most destructive subtypes of stroke and accounts for 10–15% of all strokes (Qureshi et al., 2009). Despite its importance, effective treatment remains unsatisfactory, and the underlying mechanism of ICH-induced brain damage is not completely clarified. Hemoglobin (Hb) released from extravasated erythrocytes is the largest factor or a strongly oxidizing mediator in the secondary injury of ICH (Huang et al., 2002). Hb-mediated brain injury involved in multifarious mechanisms, one of the most critical was related to matrix metalloproteinases (MMPs) activation (Tejima et al., 2007; Katsu et al., 2010).

MMPs are a family of zinc endopeptidases capable of degrading components of the extracellular or neurovascular matrix, thereby resulting in neurovascular injury (Rosenberg, 2009). MMPs, in particular MMP-9, have a pivotal function in acute brain injury after ICH, blockade of MMP activity or genetic removal of MMP-9 gene during this critical period has efficacy as a

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Abbreviations: ICH, intracerebral hemorrhage; Hb, hemoglobin; BBB, blood–brain barrier; $ONOO^-$, peroxynitrite; 3-NT, 3-nitrotyrosine; CNS, central nervous system; nNOS, neuronal nitric oxide synthase; eNOS, endothelial nitric oxide synthase; iNOS, inducible nitric oxide synthase; IR, ischemia reperfusion; MCAO, middle cerebral artery occlusion; mNSS, modified neurological severity score; NO, nitric oxide; NOS, nitric oxide synthase; RNS, reactive nitrogen species; ROS, reactive oxygen species; Sham, sham-operated animals; NF-κB, nuclear factor-κB; ZO-1, zonula occludens-1.

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therapeutic strategy for the treatment of secondary brain injury (Wang and Tsirka, 2005; Tejima et al., 2007).

In the case of ICH, released Hb from ruptured erythrocytes and subsequent byproducts including reactive oxygen species (ROS) and reactive nitrogen species (RNS) (eg: superoxide radical and nitric oxide) are dramatically increased (Katsu et al., 2010; Yang et al., 2013). Oxidative stress or superoxide triggers activation of MMP-9 after intracerebral injection of Hb into rat striata (Katsu et al., 2010). Recently, it was reported that nitric oxide (NO) derived from iNOS or nNOS also directly activates MMP-9 (Wu et al., 2011). Moreover, a growing body of evidence demonstrated that not only superoxide and NO, but also their conjunctly potent toxic metabolites — peroxynitrite ($ONOO^-$) also participate in the activation of MMPs (Gursoy-Ozdemir et al., 2004; Suofu et al., 2010; Evans et al., 2012). Our previous study demonstrated that Hb could induce abundant peroxynitrite ($ONOO^-$) formation *in vivo* (Ding et al., 2014). $ONOO^-$ characterized with strong reactivity and high diffusibility is a potent biological effector molecule (Groves, 1999), excessive production of which have destructive pathological consequences by oxidizing or nitrating proteins, lipids and DNA in various CNS orders (Pacher et al., 2007). So it was inferred that $ONOO^-$ may trigger activation of MMP-9 after Hb injection.

Currently, several metalloporphyrins reported react catalytically to decompose $ONOO^-$ and attenuate the toxic effects of $ONOO^-$ *in vivo* and *in vitro* (Misko et al., 1998; Tan et al., 2004; Thiyagarajan et al., 2004; Genovese et al., 2007). Due to their high values, $ONOO^-$ decomposition catalysts have been widely used in the field of heart, lung, liver, kidney, intestinal, and other splanchnic artery occlusion and reperfusion injury (Cuzzocrea et al., 2000; Lauzier et al., 2007; Soriano et al., 2011; Seija et al., 2012). FeTPPS, the typical member of metalloporphyrins, can isomerize $ONOO^-$ to the harmless nitrate anion (Salvemini et al., 1998). In a rat middle cerebral artery occlusion model, infarct volume, brain edema and neurological deficits were significantly decreased by FeTPPS treatment (Thiyagarajan et al., 2004). FeTPPS also effectively protected against neuronal damage by reducing oxidative stress and DNA fragmentation in global cerebral ischemic-reperfusion injury in gerbils (Sharma et al., 2007). Gursoy-Ozdemir et al. once reported that $ONOO^-$ formation on microvessels colocalizes with MMP-9 expression (Gursoy-Ozdemir et al., 2004). Recent evidence *in vivo* strongly demonstrated that $ONOO^-$ can upregulate the expression of MMP-9 (Evans et al., 2012) and its decomposition or reduction brings significantly beneficial effects (Suofu et al., 2010). *In vitro*, peroxynitrite also strongly activates MMP-1, -2, -8 and -9 (Okamoto et al., 2001; Sugiura et al., 2012), and inactivates tissue inhibitors of metalloproteinase-1 (Frears et al., 1996). However, whether FeTPPS can ameliorate the neurovascular injury and improve neurological function and if the benefits of FeTPPS attribute to the inhibition of MMP-9 activation after ICH is still unknown.

The objective of the present study was to examine the hypothesis that administration of a peroxynitrite decomposition catalyst (FeTPPS) in Hb-induced rat brain injury model, ameliorate the neurovascular dysfunction and neurological deficits, possibly in part by decreasing MMP-9 activation.

EXPERIMENTAL PROCEDURES

Animals

Male Sprague–Dawley (SD) rats, weighing approximately 300 g (ranging from 280 to 320 g), were purchased from the Animal Experiment Center of Southern Medical University (Guangzhou, China). Animal experimental procedures were approved by the Southern Medical University Ethics Committee. All surgery was performed under sodium pentobarbital anesthesia, and all efforts were made to minimize animal suffering. Animals were housed under a 12-h light/dark cycle, with food and water freely available.

Experimental groups

SD rats were divided into three groups: (1) sham group (30 rats); (2) Hb-injected + vehicle group (36 rats); (3) Hb-injected + FeTPPS group (36 rats). FeTPPS was administered (30 mg/kg, intraperitoneally) (EMD Biosciences, Inc., San Diego, CA, USA) immediately after Hb-injection and at 12 h, 36 h, 60 h (or started at 6 h and then 18 h, 42 h, 66 h). Saline was used as vehicle for FeTPPS. The dose of the FeTPPS was chosen from previous reports which showed that it had better protection against injury (Salvemini et al., 1998; Genovese et al., 2007).

Animal model

The Hb-induced brain injury model was prepared as described previously (Yang et al., 2013). Briefly, after anesthetized and positioned, an incision along the sagittal midline was made to expose the skull using a sterile technique. After the cranial burr hole (1 mm) was drilled, a microsyringe was inserted stereotactically into the right caudate nucleus (3 mm lateral to the midline, 1 mm anterior to the coronal suture of the bregma, 6 mm below the surface of the skull, and then withdrawn 0.5 mm). Using a microinjection pump, 20 μL of hemoglobin (Product number: H7379; Sigma–Aldrich, St. Louis, MO, USA) at a concentration of 150 mg/mL was injected into the nucleus over 10 min. Rats, in the sham group, were subjected to only a needle insertion in the same way. After injection, the needle was left in place for an additional 10 min to prevent any reflux and then slowly removed. The burr hole was sealed with bone wax, and the skin incision was closed. The animals were allowed to recover with free access to food and water for the duration of observation.

In situ zymography and double labeling with fluorescent probes

In situ gelatinolytic activity was detected on frozen brain sections with a thickness of 10 um using a commercial kit (Gelatinase Assay kit; GENMED, Shanghai, China)