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Role of iron in brain lipocalin 2 upregulation after intracerebral hemorrhage in rats

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ABSTRACT

Brain iron overload has a detrimental role in brain injury after intracerebral hemorrhage (ICH). Lipocalin 2 (LCN2), a siderophore-binding protein, is involved in cellular iron transport. The present study investigated changes in LCN2 expression after ICH and the role of iron in those changes. Male Sprague-Dawley rats had an intracaudate injection of autologous blood (ICH) or iron. Control rats received a needle insertion or saline injection. Some ICH animals were treated with either vehicle or deferoxamine, an iron chelator. Brain LCN2 expression was determined by Western blot analysis and immunohistochemistry. Real-time PCR was also used to confirm brain LCN2 mRNA expression. The number of LCN2 positive cells was markedly increased in the ipsilateral basal ganglia and cortex after ICH and most LCN2 positive cells were astrocytes. Western blots showed that brain LCN2 levels were higher at days 1, 3 and 7 in the ipsilateral hemisphere after ICH (70 to 80 fold higher than contralateral hemisphere or sham-operated rats at 3 days), and declined to lower levels at day 14. Iron, but not saline injection also caused brain LCN2 upregulation (a more than 100-fold increase). In addition, systemic treatment of deferoxamine reduced ICH-induced LCN2 upregulation ($p<0.05$). These results suggest that iron has a role in brain LCN2 upregulation following ICH. LCN2 upregulation after ICH may be part of the response to clear iron released from the hematoma during clot resolution.

1. Introduction

Intracerebral hemorrhage (ICH) is a devastating form of stroke with high morbidity and mortality (Qureshi et al., 2009; Rincon and Mayer, 2012). Evidence suggests that iron-overload is involved in ICH-induced brain damage resulting in perihematoma edema, neuronal death and brain atrophy (Keep et al., 2012; Xi et al., 2006). We have demonstrated that deferoxamine, an iron chelator, reduces ICH-induced brain injury in young and aged rats (Okauchi et al., 2010; Xi et al., 2006), as well as in pigs (Gu et al., 2009). Clinical studies also found that high levels of serum ferritin, an iron storage protein, are independently associated with severe brain edema and poor prognosis in ICH patients (Mehdiratta et al., 2008; Perez de la Ossa et al., 2010), suggesting a role of iron in ICH-induced brain injury (Selim et al., 2011).

Abbreviations: ICH, Intracerebral hemorrhage; LCN2, Lipocalin 2; GFAP, Glial acidic fibrillary protein

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Lipocalin 2 (LCN2), also known as neutrophil gelatinase associated lipocalin (NGAL) or 24p3, is a siderophore-binding protein. LCN2 mediates iron uptake in cells expressing the LCN2 cell surface receptor, LCN2R (24p3R) (Devireddy et al., 2005). Recently, the function of LCN2 within the CNS has started to be elucidated. *In vivo* studies have demonstrated LCN2 expression in the choroid plexus in response to peripheral lipopolysaccharide administration (Marques et al., 2008) and excitotoxic brain injury (Chia et al., 2011). Recent studies showed that LCN2 plays a detrimental role in the CNS after spinal cord injury (Rathore et al., 2011) but it appears to play a protective role in experimental autoimmune encephalomyelitis (Berard et al., 2012).

In the present study, we examined the time course of brain LCN2 expression in a rat model of ICH. The role of iron in ICH-induced LCN2 expression was also examined.

2. Results

ICH-induced changes in LCN2 were measured by immunohistochemistry and Western blotting. LCN2 positive cells were found in the ipsilateral basal ganglia but not the contralateral basal ganglia at days 1, 3, 7 and 14 after ICH (Fig. 1A). The LCN2 protein levels determined by Western blotting in the ipsilateral basal ganglia were significantly increased at day 1, remained at high levels at days 3 and 7, and the decreased markedly at day 14 after ICH (Fig. 1B). Similar results were found in the ipsilateral cortex following ICH (Fig. 1C).

LCN2 immunoreactivity was very weak in sham-operated brain but increased significantly after blood injection. LCN2 positive cells were astrocyte-like (Fig. 2A). Western blots, with LCN2 normalized to β-actin, indicated that LCN2 protein levels in the ipsilateral basal ganglia were 71-fold higher than those in the contralateral basal ganglia (LCN2/β-actin ratio: 2.70±0.46 vs. 0.04±0.01, $p<0.001$) and 84-fold higher than in sham-operated animals (0.92±0.14 vs. 0.01±0.01, $p<0.001$) at 3 days after ICH (Fig. 2B). Similar results were also found in the cortex (Fig. 2B).

Double-labeling was used to determine which types of cells express LCN2 after ICH. We found that LCN2 positive cells predominantly colocalized with glial fibrillary acid protein (GFAP) positive cells after ICH. There were only a few LCN2 positive cells that colocalized with neuronal nuclear antigen (NeuN) in cortex (Fig. 3). These results suggest that LCN2 is mainly expressed in astrocytes following ICH.

To test whether iron can induce LCN2 expression, 30 μL of ferrous chloride (1 mmol/L) or saline was injected into the rats' right basal ganglia. There were many more LCN2 positive cells in the ipsilateral basal ganglia after iron injection compared to saline injection (Fig. 4A). Iron injection caused a 136-fold increase of LCN2 protein levels at day 3 (ratio of LCN2/β-actin: 1.77±0.43 vs. 0.013±0.004 in saline injection, $p<0.01$, Fig. 4B). To examine whether ICH-induced LCN2 expression can be attenuated by iron chelation, rats were treated with deferoxamine or vehicle (2 h and then every 12 h for 7 days) after ICH. Deferoxamine reduced ICH-induced LCN2 upregulation at day 7 (ratio of LCN2/β-actin: 0.44±0.23 vs. 1.73±0.5 in vehicle, $p<0.05$, Fig. 5).

To further elucidate whether the brain produces LCN2 after ICH, quantitative real-time PCR of LCN2 gene expression was examined in triplicate using Eppendorf Mastercycler Realplex Detection System. Real-time PCR showed that LCN2 mRNA expression in the ipsilateral basal ganglia was increased 6.5-fold compared to the contralateral basal ganglia at 24 h after ICH ($p<0.05$).

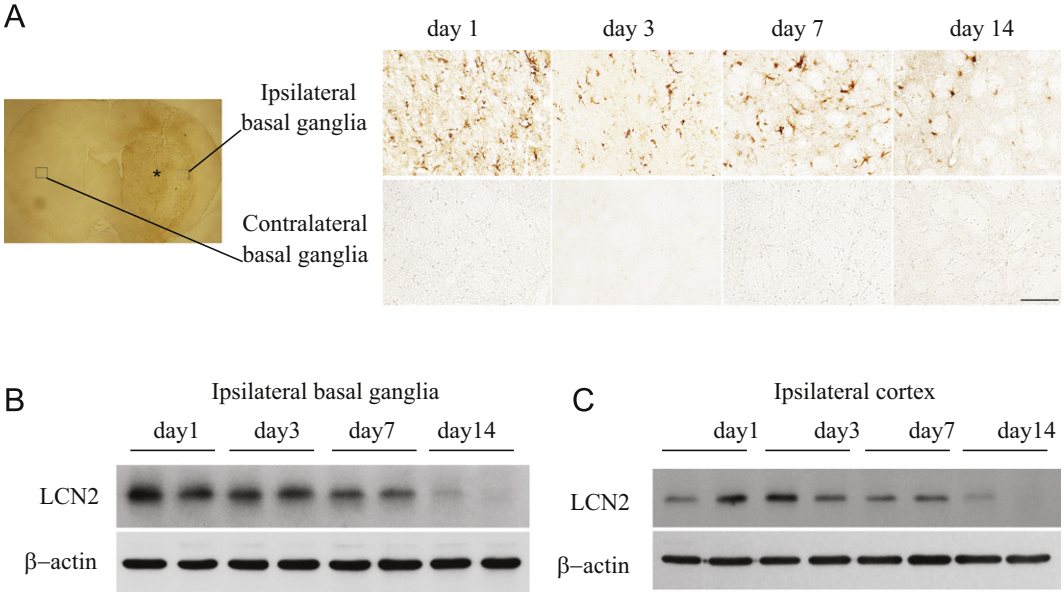

Fig. 1 – Time course showing LCN2 immunoreactivity in the ipsi- and contra-lateral basal ganglia (A), and the protein levels of LCN2 and β-actin in the ipsilateral basal ganglia (B) and cortex (C) after an injection of 100 μl autoglous blood into the right caudate. The asterisk indicates the hematoma, the boxes indicate the locations where higher powered immunohistochemistry images were taken. Scale bar = 100 μm.