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Research Report

Inhibition of receptor-interacting protein 3 upregulation and nuclear translocation involved in Necrostatin-1 protection against hippocampal neuronal programmed necrosis induced by ischemia/reperfusion injury

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ABSTRACT

Receptor-interacting protein 3 (RIP3) is a key molecular switch in tumor necrosis factor-induced necroptosis requiring the formation of an RIP3–RIP1 complex. We have recently shown that hippocampal cornu ammonis 1 (CA1) neuronal death induced by 20-min global cerebral ischemia/reperfusion (I/R) injury is a form of programmed necrosis. However, the mechanism behind this process is still unclear and was studied here. Global cerebral ischemia was induced by the four-vessel occlusion method and Necrostatin-1 (Nec-1), a specific inhibitor of necroptosis, was administered by intracerebroventricular injection 1 h before ischemia. Normally, in the hippocampal CA1 neurons, RIP1 and RIP3 are located in the cytoplasm. However, after I/R injury, RIP3 was upregulated and translocated to the nucleus while RIP1 was not affected. Nec-1 pretreatment prevented hippocampal CA1 neuronal death and I/R induced changes in RIP3. Decreased level of NAD+ in hippocampus and the release of cathepsin-B from lysosomes after I/R injury were also inhibited by Nec-1. Our data demonstrate that Nec-1 inhibits neuronal death by preventing RIP3 upregulation and nuclear translocation, as well as NAD+ depletion and cathepsin-B release. The nuclear translocation of RIP3 has not been reported previously, so this may be an important role for RIP3 during ischemic injury.

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1. Introduction

Necrosis has long been considered a type of un-programmed cell death, however, recent studies suggest some types of necrosis may actually be programmed and these are termed programmed necrosis (Proskuryakov et al., 2003; Degterev et al., 2005; Vandenabeele et al., 2012). Many types of clinical events lead to global ischemia. The brain, the hippocampal cornu ammonis 1 (CA1) layer in particular, is especially vulnerable. Hippocampal CA1 neuronal death induced by transient global cerebral ischemia and reperfusion (I/R) injury occurs in a delayed manner, and has been suggested, in a number of studies, to be caused by apoptosis (Zhao et al., 2005; Zhu et al., 2005). However, other studies based on electron microscopic observations suggest that the mechanism is through necrosis (Colbourne et al., 1999; Yamashima et al., 2003; Zhu et al., 2005). Based on previous studies in our lab we are suggesting that this type of cell death occurs through a process called programmed necrosis that encompasses both pathways (Wang et al., 2011).

Programmed necrosis induced by tumor necrosis factor (TNF) has been studied in a number of cell lines (Degterev et al., 2005; Cho et al., 2009; Zhang et al., 2009; Gunther et al., 2011; Welz et al., 2011; Sosna et al., 2013), however, results from the few in vivo studies are inconclusive. In TNF-induced necroptosis, the formation of a receptor-interacting protein 3 (RIP3)–RIP1 complex is required with RIP3 being the key molecular switch connecting apoptosis, necrosis and necroptosis (Degterev et al., 2005; Cho et al., 2009; Zhang et al., 2009; Gunther et al., 2011; Welz et al., 2011; Sosna et al., 2013). Programmed necrosis has also been reported to be involved in several different types of I/R injury (Degterev et al., 2005; Northington et al., 2011; Linkermann et al., 2012; Oerlemans et al., 2012). The protective effects of Necrostatin-1 (Nec-1), a specific inhibitor of necroptosis (Degterev et al., 2005), has been described using several models including middle cerebral artery occlusion (Degterev et al., 2005), neonatal hypoxia–ischemia (Northington et al., 2011), myocardial (Oerlemans et al., 2012) and renal I/R injury (Linkermann et al., 2012). While the formation of a RIP3–RIP1 complex has not been examined in most studies, and only one report involving myocardial I/R injury showed that enhanced RIP3–RIP1 interaction could be inhibited by Nec-1 (Oerlemans et al., 2012).

To date, however, the mechanism behind hippocampal CA1 neuronal death and the possible involvement of RIP3 following global cerebral I/R injury remains unclear. In the present study, we examined intracellular changes in RIP3 and the role of Nec-1 in global cerebral I/R injury.

2. Results

2.1. The location of RIP3 in hippocampal CA1 neurons and changes induced by 20-min global cerebral I/R injury

β-Tubulin-III is a microtubule element expressed exclusively in neuronal cytoplasm. DAPI stains nuclei in normal hippocampal CA1 neurons. RIP3 IF (red) co-localized with β-Tubulin-III (green) rather than DAPI stained nuclei (blue) (Fig. 1a). This suggests that RIP3 is located in the neuronal cytoplasm rather than the nuclei of normal cells.

RIP3 expression showed a large neuronal cytoplasmic increase beginning at 12 h and peaking at 48 h following re-perfusion after the 20-min ischemic period (here after known as post re-perfusion). RIP3 IF labeling in pyknotic neuronal nuclei with condensed DAPI labeling was observed in few neurons at 12 h following post re-perfusion, and peaked at 48 h. At 72 h post re-perfusion, the color faded and RIP3 IF dispersion was observed (Fig. 1b). To further confirm the changes observe in RIP3 IF, cytosolic and nuclear proteins were extracted and quantified by WB. In the control group, a relative faint RIP3 nuclear protein band was observed. By contrast, RIP3 cytosolic expression was up-regulated between 12 h and 72 h post re-perfusion while nuclear RIP3 increased between 12 h and 48 h with both reaching maximal levels by 48 h post re-perfusion. Nuclear RIP3 levels decreased significantly at 72 h compared to 48 h (Fig. 1c).

2.2. Nec-1 inhibits neuronal programmed necrosis, elevated expression and nuclear translocation of RIP3 after I/R injury

In Fig. 2a, normal neurons exhibit round and pale nuclei, while neurons undergoing cell death showed pyknotic or absent nuclei. At day 7 post re-perfusion, 91.83 ± 1.32% of CA1 neurons were destroyed (vehicle). Pretreatment with either 0.1 μg or 1 μg Nec-1 1 h before ischemia induction significantly reduced neuronal death after I/R injury with 1 μg Nec-1 being the most effective dose (Fig. 2b). Therefore, the 1 μg dosage was used in all subsequent experiments.

As seen previously in the control groups (Fig. 1b), RIP3 remained in the cytosol of most hippocampal CA1 neurons at various time periods post re-perfusion in rats pretreated with 1 μg Nec-1 (Fig. 3). However, elevated RIP3 expression and nuclear translocation were observed in several dying DAPI-stained neurons showing pyknotic nuclei (arrows in Fig. 3). The results suggested Nec-1 inhibit the upregulation and nuclear translocation of RIP3 in most CA1 neurons, yet it is still involved in the death of few dying neurons.

2.3. Changes in RIP1 and PARP-1 expression after I/R injury

It has been reported that RIP3 becomes functioning after complexing with RIP1, therefore, we wanted to examine the changes of RIP1. In this study, RIP1 was only detected in the cytosol not in the nucleus, and no changes were observed post re-perfusion (Fig. 4a and b). It has been reported that over activation of PARP-1 results in energy depletion leading to caspase-independent necrosis (Los et al., 2002). Herein, PARP-1 expression showed no changes in nucleus but increased in cytoplasm after I/R injury, and was significantly different at 72 h post re-perfusion (Fig. 4a and b).

2.4. Nec-1 inhibits the decrease in NAD+ and the release of cathepsin-B from lysosomes after I/R injury

Many studies have shown that the decrease of NAD+ involved in I/R injury, so it was detected in this study. The