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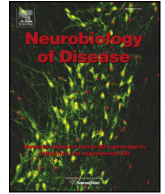

Ischemic insults induce necroptotic cell death in hippocampal neurons through the up-regulation of endogenous RIP3

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ABSTRACT

Global cerebral ischemia induces selective acute neuronal injury of the CA1 region of the hippocampus. The type of cell death that ensues may include different programmed cell death mechanisms namely apoptosis and necroptosis, a recently described type of programmed necrosis. We investigated whether necroptosis contributes to hippocampal neuronal death following oxygen-glucose deprivation (OGD), an in vitro model of global ischemia. We observed that OGD induced a death receptor (DR)-dependent component of necroptotic cell death in primary cultures of hippocampal neurons. Additionally, we found that this ischemic challenge upregulated the receptor-interacting protein kinase 3 (RIP3) mRNA and protein levels, with a concomitant increase of the RIP1 protein. Together, these two related proteins form the necrosome, the complex responsible for induction of necroptotic cell death. Interestingly, we found that caspase-8 mRNA, a known negative regulator of necroptosis, was transiently decreased following OGD. Importantly, we observed that the OGD-induced increase in the RIP3 protein was paralleled in an *in vivo* model of transient global cerebral ischemia, specifically in the CA1 area of the hippocampus. Moreover, we show that the induction of endogenous RIP3 protein levels influenced neuronal toxicity since we found that RIP3 knock-down (KD) abrogated the component of OGD-induced necrotic neuronal death while RIP3 overexpression exacerbated neuronal death following OGD. Overexpression of RIP1 also had deleterious effects following the OGD challenge. Taken together, our results highlight that cerebral ischemia activates transcriptional changes that lead to an increase in the endogenous RIP3 protein level which might contribute to the formation of the necrosome complex and to the subsequent component of necroptotic neuronal death that follows ischemic injury.

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Introduction

The brain damage due to cerebral ischemia is one of the major causes of disability in the western world. Transient global cerebral ischemia that results from a lack of blood supply to the whole brain in consequence of cardiac arrest, leads to the selective and delayed death of certain populations of neurons. The hippocampus is one of the most severely affected areas in patients (Petito et al., 1987) and also in animal models of global ischemia (Kirino, 1982; Zukin et al., 2004). Global cerebral ischemic insults can be simulated in vitro by performing oxygen-glucose deprivation (OGD) on primary neuronal cultures or slices, typically from the hippocampus or cortex (Goldberg and Choi, 1993; Martin et al., 1994; Calderone et al., 2003).

Cerebral ischemic insults both *in vivo* and *in vitro* induce necrotic as well as apoptotic neuronal death (Gwag et al., 1995; Martinez-Sanchez et al., 2004; Malagelada et al., 2005). In recent years, however, a novel

Abbreviations: *Actb*, β-Actin; cIAP, Cellular Inhibitor of Apoptosis Protein; Ctx, Cortex; CYLD, Cylindromatosis; DG, Dentate Gyrus; DISC, Death Inducing Signaling Complex; DR, Death Receptor; FADD, Fas-associated Protein with Death Domain; *Gapdh*, Glyceraldehyde 3-phosphate dehydrogenase; LDH, Lactate Dehydrogenase; MK-801, (5S,10R)-(+)-5-Methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine maleate; Nec-1, Necrostatin-1; NMDAR, N-methyl-D-aspartate Receptor; NF-κB, Nuclear Factor Kappa B; OGD, Oxygen-glucose Deprivation; PI, Propidium Iodide; RIP, Receptor-interacting Protein Kinase; SIRT2, Sirtuin2; TNFα, Tumor Necrosis Factor α; TNFR1, Tumor Necrosis Factor Receptor 1; TRAF2, TNFR Associated Factor 2; zVAD.fmk, N-Benzyloxycarbonyl-Val-Ala-Asp(O-Me)-fluoromethyl ketone.

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type of cell death, called necroptosis, has been shown to contribute to ischemic injury (Degterev et al., 2005; Xu et al., 2010; Meloni et al., 2011; Northington et al., 2011). This type of regulated necrotic cell death was described to occur as a consequence of death receptor (DR) signaling, in conditions where apoptosis is inhibited or downregulated (Fiers et al., 1995; Vercammen et al., 1998; Van Herreweghe et al., 2010; Han et al., 2011). Although the necrotic component of cell death was, for a long time, considered to be unregulated and thus irreversible, this idea has been challenged in recent years. In a context of cerebral ischemia a complete understanding of the mechanisms of regulated necrosis might provide new targets for the therapy of this neurological disorder.

Tumor necrosis factor receptor 1 (TNFR1) signaling is complex and may have distinct outcomes. Upon tumor necrosis factor α (TNFα) binding to TNFR1, the receptor becomes activated and recruits a complex of proteins (complex I) to its vicinity that comprises the receptor-interacting protein kinase 1 (RIP1) and the TNFR associated factor 2 (TRAF2). This leads to nuclear factor kappa B (NF-κB) activation followed by expression of anti-apoptotic proteins, such as cellular inhibitor of apoptosis proteins (cIAPs) (Micheau and Tschopp, 2003), among others. The NF-κB activation downstream of TNFR1 signaling is regulated by post-translational modifications of RIP1 and TRAF2. When RIP1 is deubiquitinated by cylindromatosis (CYLD) or cIAP proteins are inhibited, the proteins of complex I dissociate from the receptor allowing the association, in the cytoplasm, of the so-called complex II or death inducing signaling complex (DISC), that recruits proteins such as Fas-associated protein with death domain (FADD), procaspase-8 and RIP1 (Micheau and Tschopp, 2003). Caspase-8 then becomes active and initiates the extrinsic apoptotic pathway. In cells with high expression of RIP3, this kinase might enter complex II due to the interaction with RIP1. Caspase-8 acts as a negative regulator of necroptosis, by promoting a cleavage of RIP1 and RIP3 in complex II (Feng et al., 2007; Cho et al., 2009). Upon inhibition of apoptosis RIP1 and RIP3 are able to induce necroptosis, by forming complex IIb, or necrosome (Vercammen et al., 1998; Holler et al., 2000). While the precise mechanism by which RIP1 and RIP3 induce necroptosis is not fully understood it is known that their kinase activity is important for this process (Cho et al., 2009; He et al., 2009; Zhang et al., 2009) and recently it was shown that their interaction is regulated by sirtuin2 (SIRT2)-dependent RIP1 deacetylation (Narayan et al., 2012). Nevertheless, the events that trigger the assembly of the necrosome in the context of cerebral ischemia are not yet known.

In this work we examined the neuroprotective effect of the necroptosis inhibitor necrostatin-1 (Nec-1) on OGD-challenged hippocampal neurons and we investigated the molecular determinants underlying OGD-induced necroptosis in hippocampal neurons, namely the role of RIP3 in this process. We show that ischemic insults induced an upregulation of RIP3 mRNA and protein levels, accompanied by a transient caspase-8 mRNA downregulation. The changes in RIP3 protein level correlated with increased hippocampal neuronal death following OGD. Importantly, we also observed an increased RIP3 protein level in the CA1 region of the hippocampus of rats submitted to global cerebral ischemia *in vivo*. These results contribute to the elucidation of the mechanism of cerebral ischemia-induced necroptosis and therefore may pave the way to novel therapeutic targets for cerebral ischemia.

Results

OGD induces a component of necroptotic neuronal death

Emerging evidence suggests that necroptosis contributes to ischemic brain injury *in vivo* (Degterev et al., 2005; Xu et al., 2010; Northington et al., 2011). This evidence is mostly based on the neuroprotective effect of Nec-1, an inhibitor of necroptosis, against this type of insult. We investigated whether necroptotic neuronal death occurs when hippocampal neurons are submitted to OGD, an in vitro model of global ischemia, more amenable to the molecular dissection of cell death mechanisms. The OGD challenge consists of combining the deprivation of both oxygen and glucose, thereby mimicking the lack of blood supply that occurs during ischemia. To study the putative contribution of necroptosis for OGD-induced neuronal death, we incubated primary cultures of rat hippocampal neurons with Nec-1 or its inactive analog. Using different cell death assays we confirmed that Nec-1 had a neuroprotective effect against OGD-induced hippocampal neuronal death suggesting a component of necroptosis (Fig. 1). In fact, using the nucleic acid dyes PI and Hoechst 33342, we observed that Nec-1 (20 μM) significantly reduced necrotic neuronal death from 12.4 ± 1.0% to 8.9 ± 0.4% (Fig. 1-A) without having an effect on the apoptotic-like neuronal death component (30.4 ± 6.2% cell death on the OGD condition and 32.7 ± 4.7% with Nec-1) (Fig. 1-C). We performed this analysis by counting the number of PI positive nuclei that do not present pyknosis, which correspond to necrotic cells, while the apoptotic-like cells presented chromatin condensation with Hoechst staining. When we used the lactate dehydrogenase (LDH) assay, which is an indirect measure of membrane leak, we also detected a neuroprotective effect of Nec-1 since we observed a reduction from 38.6 ± 2.2% to 26.5 ± 2.1% of the LDH release (Fig. 1-E). Moreover, we observed that N-Benzyloxycarbonyl-Val-Ala-Asp(O-Me)-fluoromethyl ketone (zVAD.fmk — 20 μM), a broad-spectrum caspase inhibitor, affected the apoptotic component of cell death reducing the number of apoptotic-like nuclei from 30.4 ± 6.2% to 21.1 ± 2.9% (Fig. 1-C), but had no significant neuroprotective effect when we analyzed necrotic neuronal death (12.4 ± 1.0% of necrotic neurons in OGD compared to 16.5 ± 1.2% for OGD in the presence of zVAD.fmk) (Fig. 1-A). In some experiments we observed PI-positive speckles in some nuclei, similarly to what has been reported in other OGD studies (Kaasik et al., 2001; Fang et al., 2012). Additionally, we detected cytoplasmatic labeling with PI in some cells, which could be related to RNA staining, since we used PI as a vital dye and therefore did not pre-incubate neurons with RNAases.

Recently, events of DR-independent necroptosis were described (Feoktistova et al., 2011; Tenev et al., 2011), so we tested whether a TNFα neutralizing antibody could influence cell death induced by OGD. We observed that by inhibiting TNFα signaling we were able to significantly protect neurons from OGD-induced death (LDH release was reduced from 24.2 ± 1.9% to 16.3 ± 1.4%) (Fig. 1-F), while having no effect when applied in control conditions. This suggests that in neurons submitted to OGD, DR signaling may mediate the activation of necroptotic cell death.

Necroptosis in OGD-challenged neurons is promoted by up-regulation of RIP3

In recent years many efforts have been made to clarify the mechanisms underlying necroptosis (Chan and Baehrecke, 2012). However, in neurons, the mechanism by which necroptosis is activated is not known. In order to investigate the mechanism underlying the necroptotic component of neuronal death induced by OGD we analyzed the mRNA levels of two known regulators of necroptosis (Fig. 2): RIP3, a known specific necroptosis player upon adequate stimuli (Cho et al., 2009; He et al., 2009; Zhang et al., 2009) (Fig. 2-A) and caspase-8, which is a negative regulator of necroptosis (Oberst et al., 2011) (Fig. 2-B). We observed by microarray analysis that both genes were altered following OGD (data not shown) while no other caspases were changed in these conditions. Interestingly, we found that RIP3 is significantly upregulated at 7 and 24 h following OGD while caspase-8 is transiently downregulated 7 h after OGD. These results suggest that a subset of neurons may downregulate apoptosis, while upregulating RIP3 expression, which may become afterwards more available for activation of necroptotic signaling. Additionally, caspase-8 downregulation may also contribute to the relief of the negative regulation exerted by this protein on necroptotic signaling.

To confirm that RIP3 mRNA levels translate to increased protein expression following OGD, we analyzed total RIP3 levels by western blotting. Indeed, at 24 h after OGD we observed a significant increase in RIP3

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