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BRAIN RESEARCH

Research Report

Synergistic protective effects of humanin and necrostatin-1 on hypoxia and ischemia/reperfusion injury

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ABSTRACT

Since several different pathways are involved in cerebral ischemia/reperfusion injury, combination therapy rather than monotherapy may be required for efficient neuroprotection. In this study, we examined the protective effects of an apoptosis inhibitor Gly^{14}-humanin (HNG) and a necroptosis inhibitor necrostatin-1 (Nec-1) on hypoxia/ischemia/reperfusion injury. Cultured mouse primary cortical neurons were incubated with Nec-1, HNG or both in a hypoxia chamber for 60 min. Cell viability was determined by MTS assay at 24 h after oxygen-glucose deprivation (OGD) treatment. Mice underwent middle cerebral artery occlusion for 75 min followed by 24 h reperfusion. Mice were administered HNG and/or Nec-1 (i.c.v.) at 4 h after reperfusion. Neurological deficits were evaluated and the cerebral infarct volume was determined by TTC staining. Nec-1 or HNG alone had protective effects on OGD-induced cell death. Combined treatment with Nec-1 and HNG resulted in more neuroprotection than Nec-1 or HNG alone. Treatment with HNG or Nec-1 reduced cerebral infarct volume from 59.3±2.6% to 47.0±2.3% and 47.1±1.5%, respectively. Combined treatment with HNG and Nec-1 improved neurological scores and decreased infarct volume to 38.6±1.5%. In summary, we demonstrated that the combination treatment of HNG and Nec-1 conferred synergistic neuroprotection on hypoxia/ischemia/reperfusion injury *in vitro* and *in vivo*. These findings provide a novel therapeutic strategy for the treatment of stroke by combining anti-apoptosis and anti-necroptosis therapy.

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1. Introduction

Stroke is the third leading cause of death, behind heart diseases and cancer, in the United States (Rosamond et al., 2007). Intravenous administration of the tissue plasminogen activator (t-PA) is the only therapy approved by the Food and Drug Administration (FDA) for the treatment of acute ischemic stroke within 3 h of symptom onset (Anon, 1995). However, only a small percentage of patients with ischemic stroke are treated with t-PA for its narrow thera-

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Abbreviations: Nec-1, necrostatin-1; HNG, Gly^{14}-humanin; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2(4-sulfophenyl) 2-H-tetrazolium, inner salt; OGD, oxygen-glucose deprivation; TTC, 2,3,5-triphenyltetrazolium chloride; ERK, extracellular signal regulated kinase; TNFα, tumor necrosis factor-α; MCAO, middle cerebral artery occlusion; DMSO, dimethyl sulfoxide

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peutic window and contraindications of thrombolytic therapy (Wardlaw et al., 1997). Therefore, there is an urgent need for additional safe and effective treatments for ischemic stroke.

Humanin (HN) is a newly identified 24-amino acid anti-apoptotic peptide well known for its activity to suppress neuronal cell death induced by AD-related insults such as amyloid beta (Aβ) toxicity (Tajima et al., 2002). In our previous study, a highly potent HN variant Gly^{14}-HN (replacement of the 14^{th} amino acid serine with glycine, HNG) protected against cerebral ischemia/reperfusion injury in a mouse model (Xu et al., 2006a). Specifically, HNG reduced infarct volume, improved neurological deficits, and decreased the number of apoptotic neurons. The mechanism by which HNG achieves these effects involves decreasing cleaved poly(ADP-ribose)-polymerase (PARP, a marker of caspase activity), inhibiting extracellular signal regulated kinase (ERK) activation (Xu et al., 2006a), and activating PI3K/Akt signal pathways (Xu et al., 2008).

Recently, Degretev *et al* reported a novel type of cell death called necroptosis (Degterev et al., 2005). Importantly, they identified a specific necroptosis inhibitor necrostatin-1 (Nec-1) that reduced the infarct volume in a cerebral ischemia/reperfusion mouse model even when it was administered 6 h after reperfusion (Degterev et al., 2005). In a previous study, we showed that Nec-1 protects against glutamate-induced necroptosis in hippocampal HT-22 cells (Xu et al., 2007). These findings suggest that necroptosis exists in cerebral ischemia/reperfusion injury and that Nec-1 could represent a potential therapeutic intervention against this type of injury. Degterev et al. further indicated that RIP1 kinase is the cellular target for the anti-necroptosis activity of Nec-1 (Degterev et al., 2008). Our previous data also showed that Nec-1 inhibits BNIP3 translocation to inner membrane of mitochondria and indirectly blocked PARP/AIF-mediated cell death (Xu et al., 2007; Xu et al., 2010).

Since several different pathways are involved in cerebral ischemia/reperfusion injury, combination therapy rather than monotherapy may be required for efficient neuroprotection. (Gladstone et al., 2002; Grotta, 2002; Lo et al., 2003). Previous studies in animal models of stroke revealed pharmacological synergy by using two neuroprotective agents (Ma et al., 1998; Onal et al., 1997; Xu et al., 2006b). In this study, we designed a cocktail of an apoptosis inhibitor HNG and a necroptosis inhibitor Nec-1 that simultaneously acts on distinct cell death pathways *in vitro* and *in vivo*.

2. Results

2.1. *HNG and Nec-1 have synergistic protective effect on OGD-induced neuronal death in vitro*

Cultured cortical neurons were subjected to oxygen-glucose deprivation (OGD) treatment at day 10 after culture. HNG alone (0.2 μM) or Nec-1 alone (25 μM) treatment had no effect on cell viability of normal primary cortical neurons (Fig. 1). OGD-induced damage decreased cell viability to 47.9±1.1% of the control. Nec-1 or HNG treatment significantly increased cell viability to 68.4±1.6 % and 60.7±1.0 % of the control, respectively ($P<0.05$). The combination of Nec-1 and HN increased cell viability to 80.8±1.9 % of the control ($P<0.05$, versus other groups). These data indicated that Nec-1 and HNG have synergistic protective effect on OGD-induced cell death.

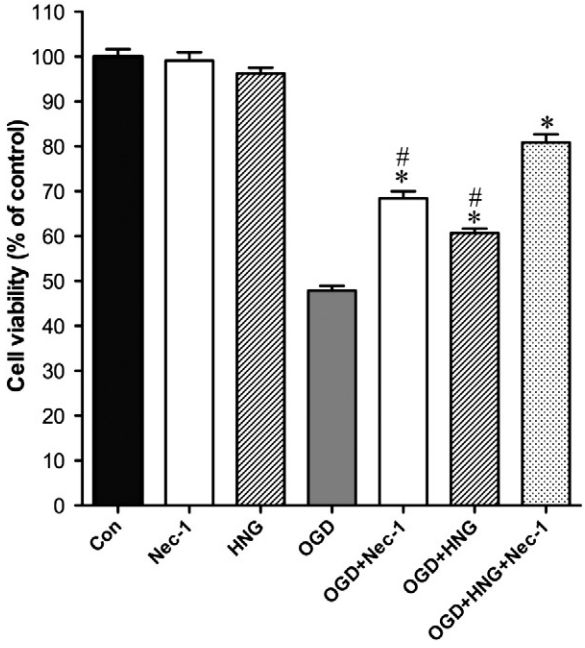

Fig. 1 – Protective effect of Nec-1 and HNG on oxygen-glucose deprivation (OGD)-induced cell death in primary cortical neurons. Primary cortical neurons were used to perform OGD at DIV 10. Neurons were washed with HBSS and incubated with vehicle, Nec-1 (25 μM), HNG (0.2 μM), or both in a hypoxia chamber for 60 min. Cell viability was determined by MTS assay 24 h after reperfusion. Control neurons were exposed to oxygenated HBSS containing 5.5 mM glucose in normoxic conditions during the same time as the OGD culture. Bars represent mean±SEM of 8 samples. *P<0.01, versus OGD group; #, P<0.01, versus OGD+HNG+Nec-1 group.

2.2. *HNG and Nec-1 have additive protection on cerebral ischemia/reperfusion injury in vivo*

In this study, we examined the protective effects of HNG and Nec-1 on cerebral ischemia/reperfusion injury. Mice were treated with HNG and/or Nec-1 at 4 h after reperfusion. This time point was chosen because our previous study showed that HNG is effective at 4 h but not 6 h after ischemia/reperfusion (Xu et al., 2006a). Neurological deficits were evaluated by a scoring system. There was no significant difference between the HNG alone or Nec-1 alone group compared with the saline-treated group ($P>0.05$ in neurological scores). However, the combination of HNG and Nec-1 treatment significantly decreased neurological deficits when compared with saline-treated vehicle group ($P<0.05$, Fig. 2). TTC staining demonstrated that HNG or Nec-1 alone reduced cerebral infarct volume from 59.3±2.6% to 47.0±2.3% and 47.1±1.5%, respectively ($P<0.05$, Fig. 3). HNG and Nec-1 treatment decreased the infarct volume to 38.6±1.5%, which