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Research Report

Role of PI3K/Akt in diazoxide preconditioning against rat hippocampal neuronal death in pilocarpine-induced seizures

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ABSTRACT

Diazoxide (DZ), a highly selective opener of the mitochondrial ATP-sensitive potassium (mitoK$_{ATP}$) channel, has neuroprotective effects. However, the mechanism of DZ protecting hippocampal neurons against cell death in pilocarpine-induced seizures is unknown. In this study, we investigated DZ attenuating neuronal loss caused by pilocarpine-induced seizures in rat hippocampus. DZ inhibited seizure-induced change in phospho-Akt expression, translocation of apoptosis-inducing factor (AIF), release of cytochrome c (CytC) and caspase-3 activation, which could be abolished by preincubation with 5-hydroxydecanoic acid, an inhibitor of mitoK$_{ATP}$. In addition, wortmannin, an inhibitor of phosphatidylinositol-3-kinase (PI3K), attenuated the translocation of AIF, CytC release and caspase-3 activation after seizures. DZ could reduce neuronal death induced by seizures in hippocampus by suppressing the translocation of AIF, CytC release and the activation of caspase-3 via the PI3K/Akt pathway.

1. Introduction

The mitochondrial ATP-sensitive potassium (mitoK$_{ATP}$) channel was reported to be involved in cerebral (Mayanagi et al., 2007; Watanabe et al., 2008) and myocardial (Pell et al., 1997; Shinohara et al., 2007) ischemic preconditioning. Moreover, the level of mitoK$_{ATP}$ channels is at least six-fold higher in brain than heart cells (Bajgar et al., 2001), which implies that mitoK$_{ATP}$ channels may play an essential role in the function of the central nervous system. ATP-sensitive potassium (K$_{ATP}$) channels are involved in several physiological functions (Yamada and Inagaki, 2005). They open and close in response to changes in intracellular ATP/ADP ratios. Low ATP levels open the channels, thus allowing K^+ efflux and cell hyperpolarization (Faraci and Sobey, 1998). Recently, the expression of functional K$_{ATP}$ channels was found to inhibit seizure responses and possibly limit the release of excitatory

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Abbreviations: DZ, diazoxide; MitoK$_{ATP}$, mitochondrial ATP-sensitive potassium; AIF, apoptosis-inducing factor; CytC, cytochrome c; 5-HD, 5-hydroxydecanoic acid; WTN, wortmannin; PI3K, phosphatidylinositol-3-kinase; SE, status epilepticus; CA3, cornu ammonis region 3

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neurotransmitters such as glutamate (Soundarapandian et al., 2007). For epilepsy, mitoK$_{ATP}$ channel openers, particularly diazoxide (DZ), protects against status epilepticus-induced neuron damage during diabetic hyperglycemia (Huang et al., 2010). However, the molecular mechanisms of the neuroprotective effect of DZ have not been fully elucidated.

Phosphatidylinositol-3-kinase (PI3K) generates phosphatidylinositol triphosphate, which activates the serine/threonine kinase Akt. Akt (also known as protein kinase B) modulates cellular activation, inflammatory response, and apoptosis (Cantley, 2002; Fruman and Cantley, 2002; Shi et al., 2007; Zhang et al., 2008). Phospho-Akt, as activation of Akt and its downstream molecules, has a role in cytoprotection. MitoK$_{ATP}$ channel openers such as DZ may activate the PI3K/Akt pathway in anoxia-reoxygenation injury (Liu et al., 2010). Mitochondria, as the downstream target of Akt activation, may cause phosphorylation of various proteins in the mitochondria (Bijur and Jope, 2003). The eventual outcome is reduced translocation of apoptosis-inducing factor (AIF) and release of cytochrome c (CytC), thus causing less apoptosis. AIF is a novel apoptotic factor that induces chromatin condensation and large-scale DNA fragmentation. AIF is a caspase-independent mitochondrial death factor (Kim et al., 2003). Release of the mitochondrial electron transport chain protein CytC into the cytosol activates caspases, thus culminating in DNA fragmentation and cytolysis (Liu et al., 1996). We postulate that the mitoK$_{ATP}$ channel is upstream of PI3K/Akt pathway and that activation of the former leads to neuroprotection.

In this study, we examined the effect of DZ on neuronal loss after pilocarpine-induced seizures in rats. Specifically, we tested whether DZ could induce Akt phosphorylation and thereafter inhibit the release of AIF and CytC and the activation of caspase-3, which has been previously shown (Zhang et al., 2010). To clarify the relationship between PI3K/Akt activation and neuroprotection by DZ after seizures, we examined the effect of wortmannin (WTN) on activity of phospho-Akt, AIF, CytC and caspase-3 proteins. We thus investigated a potential target for prevention of hippocampal neuronal damage caused by pilocarpine-induced seizures.

2. Results

2.1. *Effect of DZ on neuronal loss in hippocampus after seizures*

Nissl staining was used to examine neuronal loss in the hippocampal CA3 region after pilocarpine-induced seizures in rats; seizures led to cell death at 24 h. The number of surviving neurons with pilocarpine treatment was decreased significantly as compared with controls. Moreover, DZ pretreatment significantly attenuated the neuronal loss induced by seizures, and the protective effect was partially blocked by preconditioning with 5-hydroxydecanoic acid (5-HD) (Fig. 1; Table 1).

2.2. *DZ blocks seizure-induced change in phospho-Akt level through the mitoK$_{ATP}$ channel pathway*

We examined the level of phospho-Akt (Ser-473) at 2, 8, 16, 24 and 72 h in rat hippocampus after seizures. Phospho-Akt was increased at 2 h and then decreased thereafter (Fig. 2A, B). DZ pretreatment sharply upregulated the phospho-Akt level at 24 h after seizures as compared with pilocarpine treatment. In addition, 5-HD or WTN treatment reversed DZ-mediated Akt phosphorylation after seizures (Fig. 2C, D). Only the representative Western blots of them are illustrated in Fig. 2.

2.3. *DZ blocks seizure-induced changes in AIF translocation, CytC release and caspase-3 activation*

AIF translocated from mitochondria to the nucleus in the hippocampus after seizures beginning at 2 h and peaking at 24 h after seizures (Fig. 3A, B). Consistent with nuclear translocation, AIF level decreased in the mitochondrial fraction (Fig. 3A, B). As well, CytC level increased in the cytosolic fraction and decreased in the mitochondrial fraction after seizures (Fig. 3A, C). Moreover, the active cleavage product of caspase-3 appeared at 24 h after seizures and increased until 72 h (Fig. 2A, B). Pretreatment with DZ significantly suppressed the translocation of AIF (Fig. 3D, E), CytC release (Fig. 3D, F) and caspase-3 activation (Fig. 2C, D) at 24 h after seizures, which was counteracted by 5-HD treatment (Figs. 2 and 3).

2.4. *PI3K inhibition abolishes the effect of DZ on AIF translocation, CytC release and caspase-3 activation after seizures*

Inhibiting PI3K activation by WTN increased the translocation of AIF in the nuclear fraction (Fig. 3D, E), CytC activation in the cytosolic fraction (Fig. 3D, F) and caspase-3 activity (Fig. 2C, D) as compared with DZ treatment alone (Figs. 2 and 3).

3. Discussion

Continuous seizures can trigger neuronal degeneration in brain regions. Although great progress has been made in elucidating cell death after seizures, the mechanisms underlying neuronal death have not been studied well. Moreover, strategies to protect neuron death are still limited. Recently, DZ, a highly selective opener of the mitoK$_{ATP}$ channel, was reported to have neuroprotective effects on brain injury induced by cerebral ischemia/reperfusion (He et al., 2008). Our research in rats demonstrated massive neuronal loss in the vulnerable CA3 hippocampus area at 24 h after seizures, and DZ could significantly attenuate neuronal loss induced by seizures, as was previously described (Huang et al., 2010). Studies proved that the protective mechanisms of the mitoK$_{ATP}$ channel may involve several aspects, including Bcl-2-dependent mechanism, inhibiting the mitochondrial permeability transition pore and so on (Wu et al., 2006, 2010). Whereas, the specific mechanism by which the activation of mitoK$_{ATP}$ channel exerts protection of cell death has not yet been fully established.

We aimed to gain insight into apoptosis-related proteins and the signaling pathway modulated by DZ. We found that the translocation of AIF to the nucleus, the release of CytC from mitochondria and caspase-3 activation increased after seizures, which agrees with previous results (Wang et al.,