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Potential roles of PI3K/Akt and Nrf2–Keap1 pathways in regulating hormesis of Z-ligustilide in PC12 cells against oxygen and glucose deprivation

Hongyi Qi [a], Yifan Han [b], Jianhui Rong [a,*]

[a] School of Chinese Medicine, Li Ka Shing Faculty of Medicine, The University of Hong Kong, 10 Sassoon Road, Pokfulam, Hong Kong, PR China
[b] Department of Applied Biology and Chemical Technology, Institute of Modern Medicine, The Hong Kong Polytechnic University, Hong Kong, PR China

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ABSTRACT

Many phytochemicals may ameliorate neurological disorders through a hormetic mechanism. The aim of this study was to characterize the hormetic role of Z-ligustilide in PC12 cells against oxygen glucose deprivation (OGD) induced cell death. We examined the interactions of Z-ligustilide with the pro-survival signals mediated by phosphatidylinositol 3-kinase (PI3K) and transcription factor nuclear factor-erythroid 2 p45-related factor 2 (Nrf2) pathways. We also investigated the effect of Z-ligustilide on the intracellular redox signaling system involving reactive oxygen species (ROS) and glutathione (GSH). Z-ligustilide not only triggered stress response by causing ROS formation and transient GSH depletion, but also activated survival-promoting signals via cross-talking of PI3K and Nrf2 pathways. A key finding was that Z-ligustilide preconditioning protected PC12 cells from OGD-induced injury either at a low concentration for a prolonged period of time or at a high concentration for a short period of time. Presumably, mild preconditioning stimulated moderate ROS production, but effectively activated hormetic signals and induced stress responsive genes. In contrast, higher concentrations of Z-ligustilide could be toxic over a prolonged period of time due to massive ROS production. These results suggest that the effect of Z-ligustilide may be regulated by a biphasic hormetic mechanism involving initial induction of oxidative stress and subsequent activation of stress response gene expression.

1. Introduction

Oxidative damage of neurons in ischemic stroke is the predominant cause of mortality and chronic disability worldwide (Chan, 1996; Kato and Kogure, 1999). The development of tolerance in neuronal cells against ischemia reperfusion injury is a current therapeutic target for drug discovery (Gupta et al., 2010; Mattson and Cheng, 2006; Stankowski and Gupta, 2010). Neurohormesis, referring to hormesis in neurons, defines a class of useful stressors that could significantly enhance the neuronal resistance to more severe stress that could be lethal or cause dysfunction or disease (Mattson and Cheng, 2006). Such stressors could be endogenous neurotoxic molecules, including nitric oxide (Huang, 2004), carbon monoxide (Dore et al., 2000), glutamate (Jiang et al., 2005) and Ca^{2+} (See et al., 2001), or exogenous stimuli, such as ischemic preconditioning (Kirino, 2002), moderate-intensity excitatory stimulation (Jiang et al., 2003), exercise (Ding et al., 2004), and dietary restriction (Mattson, 2005). Interestingly, various phytochemicals are well-known to interact with the hormetic pathways to enhance an adaptive cellular stress response (Mattson and Cheng, 2006). Among multiple hormetic mechanisms, the nuclear factor E2-related factor (Nrf2)–Keap1 pathway is likely to be the central antioxidant signaling mechanism (Maher and Yamamoto, 2010; Mattson et al., 2007). The Nrf2 pathway orchestrates the different intracellular signals transduced by G-protein-coupled receptors, growth factor receptors, insulin receptors and the calcium ion channel (Mattson and Cheng, 2006). On the other hand, phosphatidylinositol 3-kinase (PI3K) promotes cell survival through the activation of not only Akt phosphorylation but also the nuclear translocation of Nrf2 (Brunet et al., 2001; Datta et al., 1999; Nakaso et al., 2003). To evaluate the pharmacological potential of various synthetic or phytochemicals, a rat pheochromocytoma cell line PC12 under oxygen glucose deprivation (OGD) was recently introduced as an *in vitro* model of ischemic stroke (Hillion et al., 2005; Kobayashi and Yamamoto, 2005; Nakajima et al., 2009; Zhou et al., 2001). A panel of neuroprotectants has been shown to enhance the cellular tolerance against oxidative insults via the activation of the hormetic pathways involving PI3K/Akt and Nrf2–Keap1 pathways (Garcia-Segura et al., 2006; Li et al., 2007; Marin et al., 2005).

* Corresponding author. Tel./fax: +852 25890537.
E-mail addresses: jrong@hku.hk, jrong@hkucc.hku.hk (J. Rong).

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Z-ligustilide is the major bioactive phthalide isolated from medicinal herbs *Rhizoma Chuanxiong* and *Radix Angelicae Sinensis*, which are widely used to treat various inflammatory diseases including stroke and myocardial infarction (Peng et al., 2007; Qi et al., 2010; Rong et al., 2008, 2007). Z-ligustilide and related phthalides were recently shown to enhance the resistance of various cell types to oxidative cell damage and promote the proliferation of rat mesenchymal stem cells (MSCs) (Qi et al., 2010; Zeng et al., 2008). The bioactivities of phthalides appear to be highly dependent on their structures. Z-ligustilide bears a typical electrophilic unsaturated lactone structure (Fig. 1A) and could directly react with Keap1 protein, causing the dissociation of Keap1–Nrf2 complex and the nuclear translocation of Nrf2 (Dietz et al., 2008). As a result, Z-ligustilide antagonized a lipopolysaccharide-induced pro-inflammatory response in primary rat microglia (Chao et al., 2010; Wang et al., 2010). Other structurally related phthalides have shown different activities. For example, senkyunolide A stimulated the degradation of tumor necrosis factor alpha (TNF-α) mRNA by an undefined mechanism other than via regulating mitogen-activated protein kinases (MAPK) and NF-κB pathways. Z-3-Butylidenephthalide was identified as an a-glucosidase inhibitor (Brindis et al., 2010). We also demonstrated that senkyunolide-H and-I provided cytoprotection against H_2O_2-induced cell death via sequential activation of the Nrf2–Keap1 pathway and induction of heme oxygenase-1 (HO-1) expression (Qi et al., 2010). Notably, HO-1 is a representative Nrf2 target gene product that catalyzes the degradation of prooxidant heme to yield biliverdin, carbon monoxide and free iron (Bauer and Bauer, 2002; Maines, 1997). The antioxidant potential of HO-1-generated metabolic products highlights the HO-1 pathway as a therapeutic target for pharmacological intervention of various diseases including neurological disorders (Maines, 1997; Prawan et al., 2005; Ryter and Choi, 2005). Among various HO-1 inducers, Z-ligustilide is a lipophilic small molecule and has been proven to be able to penetrate the blood brain barrier (Guo et al., 2009, 2011). Thus, we hypothesize that Z-ligustilide may promote the survival of PC12 cells against oxidative stress via activating hormetic pathways such as Nrf2–Keap1 and/or PI3K/Akt.

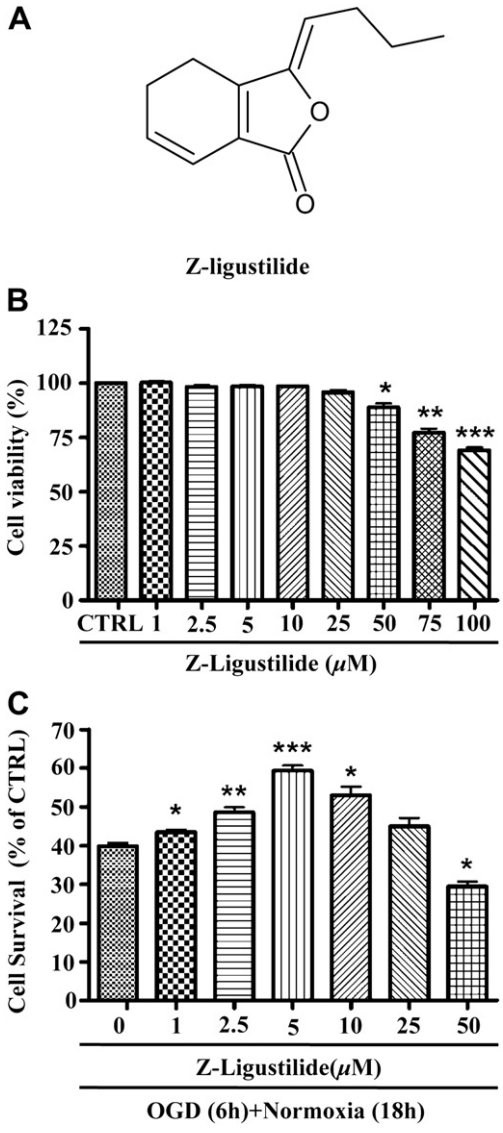

Fig. 1. Cytotoxicity and protective activity of Z-ligustilide in PC12 cells. (A) Chemical structure of Z-ligustilide. (B) Cytotoxicity of Z-ligustilide. PC12 cells were treated with Z-ligustilide at concentrations ranging from 0 to 100 μM for 24 h. The cell viability was determined by MTT assay. Values represent mean ± SD ($n = 3$). *$p < 0.05$; **$p < 0.01$ and ***$p < 0.001$ versus untreated samples (CTRL). (C) Effect of Z-ligustilide on the cell survival of PC12 cells against OGD. PC12 cells were pre-treated with Z-ligustilide at concentrations ranging from 0 to 50 μM for 18 h, subsequently subjected to OGD treatment for 6 h, and finally maintained in oxygenated cell culture medium for another 18 h. At the end of treatment, the cell viability was determined by MTT assay. Values represent mean ± SD ($n = 3$). *$p < 0.05$; **$p < 0.01$ and ***$p < 0.001$ versus OGD treated samples.

In the present study, we determined the hormetic role of Z-ligustilide on PC12 cells against oxygen glucose deprivation-induced cell death. To clarify the potential molecular mechanisms, we investigated the effect of Z-ligustilide on HO-1 induction and the potential interactions with transcriptional factor Nrf2 and signaling molecules including PI3K/Akt, ERK, JNK and p38 MAPKs by Western blot analysis using specific antibodies. We also developed a preconditioning procedure for optimizing hormetic action of Z-ligustilide against oxidative damage in PC12 cells.

2. Materials and methods

2.1. Chemicals and antibodies

Z-ligustilide was obtained from the Hong Kong Jockey Club Institute of Chinese Medicine (Hong Kong, China) with a purity of >98%. HO-1 antibody was purchased from Stressgene (Assaydesigns, Ann Arbor, MI, USA). Tin protoporphyrin (SnPP) was obtained from Porphyrin Products (Logan, UT, USA). The antibodies against β-actin and rabbit IgG were purchased from Sigma–Aldrich (St. Louis, MO, USA). The antibodies against phospho-Akt and Akt were purchased from Cell Signaling Technology (Boston, MA, USA). The antibodies against glycogen synthase kinase-3β (GSK-3β), phospho-GSK-3β, Nrf2 and lamin b were purchased from Santa Cruz Biotechnology (CA, USA). D-Cysteine was purchased from Beijing Redwood fine chemical Co. (Beijing, China). D-NAC was synthesized according to the method described by Sheffner et al. (Sheffner et al., 1966). Other chemicals were obtained from Sigma–Aldrich Co. (St. Louis, MO, USA) unless indicated otherwise.

2.2. Cell culture

Rat pheochromocytoma PC12 cells were obtained from the American Type Cell Culture Collection (Manassas, VA) and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% horse serum (Invitrogen, USA), 5% fetal bovine serum (FBS) (Invitrogen, USA), and 1% penicillin/streptomycin (Invitrogen, USA) on collagen I-coated dishes at 37 °C in a humidified 5% CO_2 atmosphere.

2.3. Measurement of cell viability

Cell viability was evaluated by a standard colorimetric assay for mitochondrial reductase catalyzed reduction of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) (Rong et al., 2007). Briefly, at the end of drug treatment, the cell monolayer was incubated in MTT solution (0.5 mg/ml) in phosphate-buffered saline (PBS) for 4 h. The formation of purple formazan was quantified by measuring the absorbance at 570 nm on a microplate reader (Bio-Rad, USA).

2.4. Procedure of oxygen glucose deprivation and normoxia treatment

The *in vitro* ischemia-reperfusion model was set up by OGD-normoxia treatment of PC12 cells as described previously (Li et al., 2008; Ye et al., 2009). Briefly, PC12