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Totarol prevents neuronal injury in vitro and ameliorates brain ischemic stroke: Potential roles of Akt activation and HO-1 induction

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ABSTRACT

The natural product totarol, a phenolic diterpenoid and a major constituent isolated from the sap of *Podocarpus totara*, has been reported to have a potent antimicrobial activity. In this study, we determined whether totarol possessed an additional neuroprotective activity in vitro and in vivo. We found that totarol prevented glutamate- and oxygen and glucose deprivation-induced neuronal death in primary rat cerebellar granule neuronal cells and cerebral cortical neurons. Totarol increased Akt and GSK-3β phosphorylation, Nrf2 and heme oxygenase-1 (HO-1) protein expressions and suppressed oxidative stress by increasing GSH and SOD activities. The PI3K/Akt inhibitor LY294002 prevented totarol neuroprotective effect by suppressing the totarol-induced changes in HO-1 expression and the activities of GSH and SOD. The HO-1 inhibitor ZnPPIX also prevented totarol-increased GSH and SOD activities. In a model of acute cerebral ischemic injury in Sprague–Dawley rats, produced by occlusion of the middle cerebral artery for 2 h followed by 22 h or 46 h of reperfusion, totarol significantly reduced infarct volume and improved the neurological deficit. In this model, totarol increased HO-1 expression and the activities of GSH and SOD. These observations suggest that totarol may be a novel activator of the Akt/HO-1 pathway protecting against ischemic stroke through reduction of oxidative stress.

1. Introduction

Ischemic stroke is a common cause of adult disability and death worldwide (Donnan et al., 2008; Pandian et al., 2007). Excessive oxidative stress is an important pathogenic mechanism in ischemic stroke. Major reductions of endogenous antioxidative systems increase excessive production of free radicals, inducing lipid peroxidation, proteins and nucleic acid oxidation (Candelario-Jalil, 2009; Granger et al., 1986). Therefore, therapeutic strategies against oxidative stress may be feasible for the treatment of ischemic stroke.

Protein kinase B (PKB, also known as Akt) and glycogen synthase kinase 3β (GSK-3β) are both serine/threonine kinases. Akt is a key kinase suppressing GSK-3β activity, which pathway activation has been reported to show beneficial effects in ischemic brain injury (Zhang et al., 2012; Zhao et al., 2014). Evidences suggest that phosphatidylinositol 3-kinase (PI3K)/Akt pathway also regulates the activity of nuclear factor erythroid 2-related factor 2 (Nrf2), which plays a key role in regulating cellular antioxidant systems and maintaining redox homeostasis (Ishii et al., 2000; Kaspar et al., 2009). Under normal conditions, Nrf2 interacts with Kelch-like ECH-associated protein 1 (Keap1) which limits Nrf2-mediated gene expression in the cytosol. Under conditions of oxidative

Abbreviations: Akt, protein kinase B; ARE, antioxidant response element; BSA, bovine serum albumin; CCA, common carotid artery; cDNA, complementary deoxyribonucleic acid; CGCs, cerebellar granule cells; CNS, central nervous system; DMEM, Dulbecco's modified eagle medium; DNase I, deoxyribonuclease I; EBSS, Earle's balanced salt solution; ECA, external carotid artery; Eda, edaravone; FBS, fetal bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GSH, glutathione; GSK-3β, glycogen synthase kinase 3β; HBSS, Hank's balanced saline solution; HO-1, heme oxygenase-1; ICA, internal carotid artery; Keap1, Kelch-like ECH-associated protein 1; LDH, lactate dehydrogenase; MCAO, middle cerebral artery occlusion; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Nrf2, nuclear factor erythroid 2-related factor 2; OGD, oxygen and glucose deprivation; PI3K, phosphatidylinositol-3-kinase; PVDF, polyvinylidene difluoride; qPCR, quantitative real-time polymerase chain reaction; RNA, ribonucleic acid; ROS, reactive oxygen species; SD, Sprague–Dawley; SOD, superoxide dismutase; TBST, Tris-buffered saline plus Tween-20; tMCAO, transient middle cerebral artery occlusion; Tot, totarol; TTC, 2,3,5-triphenyltetrazolium chloride; ZnppIX, zinc protoporphyrin-IX.

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stress, the Keap1–Nrf2 complex is dissociated, Nrf2 translocates and accumulates in the nucleus and together with small Maf proteins binds to antioxidant response element (ARE) regions in the promoter of Nrf2-mediated genes, such as heme oxygenase-1 (HO-1) (Kang et al., 2005; Kobayashi and Yamamoto, 2005).

HO-1, a ubiquitous and redox-sensitive inducible stress protein that can degrade heme to CO, iron, and biliverdin (Stocker et al., 1987), is a ARE-dependent transcription of phase II enzyme with potent antioxidant effects (Kim et al., 2011). HO-1 may regulate the antioxidant substance glutathione (GSH) and superoxide dismutase (SOD) (Gonzales et al., 2006; Lecube et al., 2014), suggesting that HO-1 may play an important role in suppressing neuronal injury.

Totarol, a phenolic diterpenoid (Fig. 1A), is a major constituent isolated from the sap of *Podocarpus totara* which is a native tree in New Zealand (Bendall and Cambie, 1995). It is well-known that totarol exhibits antiplasmodial (Clarkson et al., 2003), antimicrobial (Muroi and Kubo, 1996), and antifungal activities (Yamaji et al., 2007). However, whether totarol possesses neuroprotective effects has not yet been described.

In this study, we determined totarol neuroprotective effects and the relevant underlying mechanisms. Our results indicated that totarol exhibits neuroprotective effects through activating the Akt/HO-1 pathway, further increasing antioxidant GSH and SOD levels to suppress ischemia-induced brain injury.

2. Materials and Methods

2.1. Materials

Cell culture medium and supplements were obtained from Invitrogen (Carlsbad, CA, USA). Totarol, edaravone, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 2,3,5-triphenyltetrazolium chloride (TTC), trypsin, poly-L-lysine, LY294002, MK-2206, and zinc protoporphyrin-IX (ZnPPIX) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Primary antibodies against Nrf2 (C-20) and β-actin (AC-15) were products of Santa Cruz Biotechnology (Santa Cruz, CA, USA). An antibody against HO-1 was purchased from Abcam (Cambridge, UK). The phospho-Akt (Ser473) and the phospho-

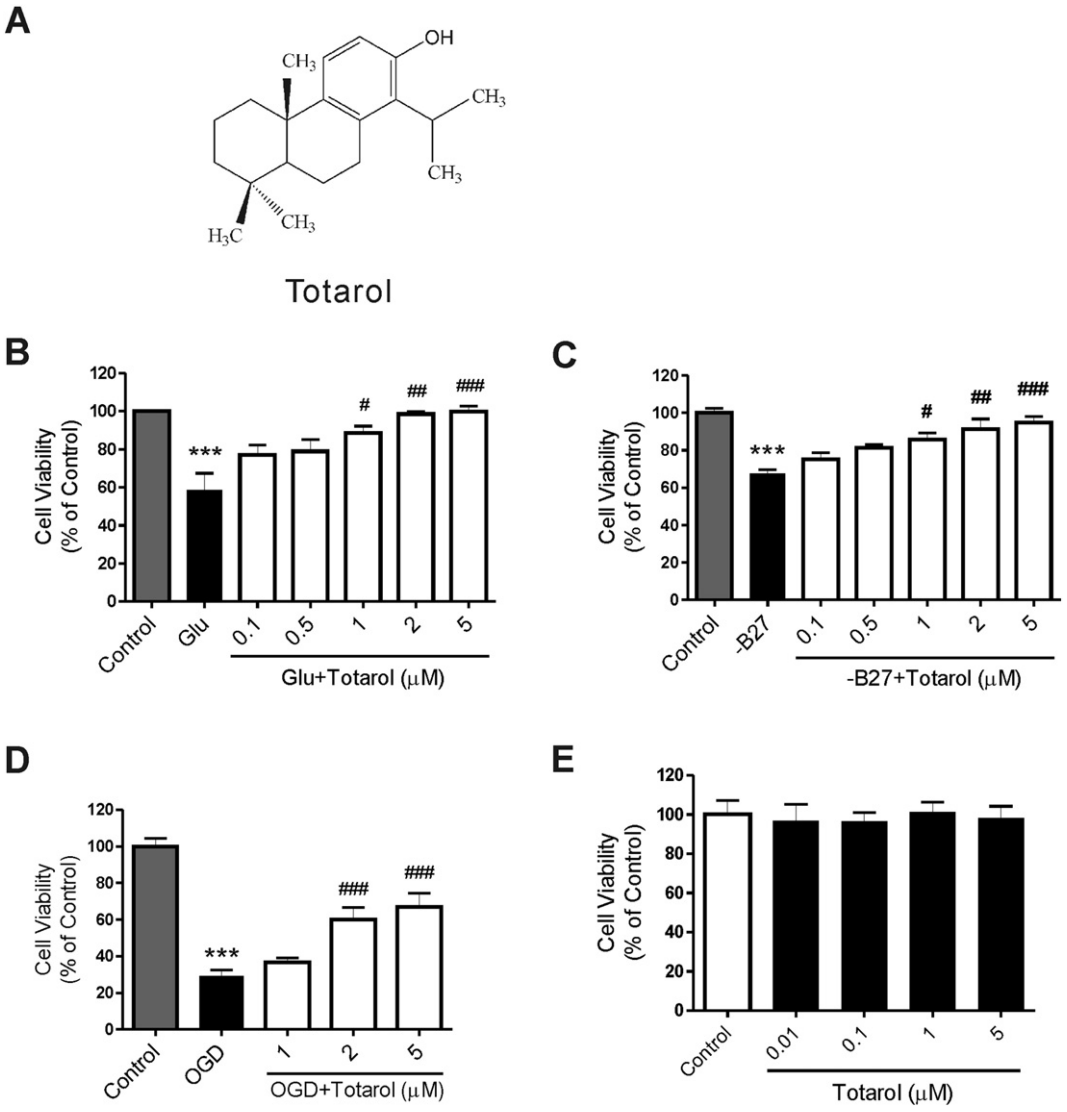

Fig. 1. Totarol prevents neurotoxicity in primary rat cerebellar granule neuronal cells (CGCs). (A) Structure of totarol (14-isopropyl-8,11,13-podocarpatrien-13-ol). (B–E) Cell viability analysis. CGCs were pretreated with various concentrations of totarol for 24 h, followed by incubation with 200 μM glutamate (Glu) for an additional 24 h (B), or followed by B27 deprivation (−B27) for an additional 24 h (C), or followed by OGD condition (D). CGCs were incubated with various concentrations of totarol for 72 h to investigate the cytotoxicity of totarol (E). Cell viability was detected using MTT assay. Results are means ± SD of at least three independent experiments. $^{***}P < 0.001$ versus control; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ versus Glu or −B27 or OGD.