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Research Report

Neuroprotective effect of nobiletin on cerebral ischemia–reperfusion injury in transient middle cerebral artery-occluded rats

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ABSTRACT

Nobiletin, a citrus polymethoxylated flavone, is reported to possess various pharmacological activities such as anticancer, anti-inflammation, and antioxidant effects. Recently, nobiletin was shown to provide therapeutic benefit for the treatment of Alzheimer's disease by activating cAMP-response element-binding protein (CREB). In the present study, we investigated whether nobiletin could protect the brain against ischemia–reperfusion (I/R) injury and improve functional outcome in cerebral I/R model rats, since CREB activation is known to protect neuronal cells in cerebral ischemia. Nobiletin was injected twice at 0 and 1 h after the start of reperfusion in transient middle cerebral artery occlusion (t-MCAO) rats. Cerebral I/R induced prominent brain damage in the ischemic hemisphere of t-MCAO rat brains; however, nobiletin treatment significantly reduced the infarct volume and suppressed the brain edema. Immunohistochemical and TUNEL staining indicated that nobiletin treatment significantly suppressed neutrophil invasion into the

Abbreviations: CREB, cAMP-response element-binding protein; I/R, HCO-60, polyoxyethylene (60) hydrogenated castor oil; MPO, myeloperoxidase; I/R, ischemia–reperfusion; t-MCAO, transient middle cerebral artery occlusion; TG, TokyoGreen; TTC, 2,3,5-triphenyltetrazolium chloride

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ischemic region and significantly decreased apoptotic brain cell death in ischemic hemisphere, suggesting that the anti-inflammatory effect and anti-apoptotic effect should be regarded as the neuroprotective mechanism of nobiletin. Moreover, nobiletin treatment ameliorated motor functional deficits in the ischemic rats compared with those deficits of the vehicle-treated group. These results indicate that nobiletin is a potential neuroprotectant for the treatment of cerebral I/R injury.

1. Introduction

Ischemic stroke is caused by a reduction in the blood supply to a part of the brain, resulting in fatal brain damage. Thrombolysis with tissue plasminogen activator (t-PA) is approved in many countries for the treatment of ischemic stroke (Young et al., 2007). Although this therapy leads to the temporal survival of cerebral cells in the ischemic region by recovering the oxygen and nutrient supply, a secondary impairment, namely, cerebral ischemia/reperfusion (I/R) injury, often occurs after recovery from ischemia (Eltzschig and Eckle, 2011; Wong and Crack, 2008). This injury is a complex disorder caused by oxidative damage, inflammation, glutamate neurotoxicity, and cerebral edema (Gursoy-Ozdemir et al., 2004; Huang et al., 2006). The suppression of brain damage from this injury is essential to obtain a good stroke outcome and to prevent a decrease in the quality of life of stroke patients. However, a therapeutic strategy for cerebral I/R injury has not been established yet (Ginsberg, 2009; Hishida, 2007; Tuma and Steffens, 2012).

Nobiletin, a flavonoid present in the peel of citrus fruits, possesses several biological activities (Nakajima et al., 2007; Onozuka et al., 2008; Matsuzaki et al., 2008; Ishiwa et al., 2000; Kandaswami et al., 1991). We previously reported that intraperitoneal treatment with nobiletin for 7 consecutive days suppresses neuronal cell death induced by 20 min ischemia in the mouse hippocampus (Yamamoto et al., 2009). Moreover, learning memory deficits following 5 min ischemia is improved by the consecutive treatment with nobiletin through stimulated phosphorylation of calcium/calmodulin-dependent protein kinase II (CAMK II) and cyclic-AMP-responsible-element-binding protein (CREB). Another report demonstrated that nobiletin shows an anti-neuroinflammatory effect by suppressing microglial activation in a microglial cell culture model (Cui et al., 2011). In addition pretreatment with nobiletin decreases H_2O_2-induced cytotoxicity in PC12 cells by several mechanisms, including those causing an increase in superoxide dismutase and glutathione activity (Lu et al., 2010). Thus, nobiletin acts as an antioxidant. In light of the above facts, nobiletin has the potential to improve cerebral I/R injury by these multiple mechanisms.

In the present study, we assessed the therapeutic effect of intravenously injected nobiletin on cerebral I/R injury as an adjunctive drug after t-PA treatment in cerebral I/R model rats, namely, transient middle cerebral artery occlusion (t-MCAO) rats. The intravenous injection of drugs is considered as a suitable route of administration for the treatment of cerebral I/R injury both because neuronal cell death progresses rapidly after I/R and because stroke patients cannot be administered drugs orally. However, nobiletin is hard to dissolve in water because of its hydrophobic property. Therefore, polyoxyethylene hydrogenated castor oil 60 (HCO-60) was used as the vehicle for nobiletin in this study. HCO-60 is an emulsifier for the formulation of some drugs and is employed clinically (Hanawa et al., 2003; Ali et al., 2010). Also, some studies have shown that HCO-60 has no effect on the outcome of ischemia in animal stroke models (Sharkey and Butcher, 1994; Zhang et al., 2005).

2. Results

2.1. *Nobiletin reduced cerebral damage in t-MCAO rats*

At first, we investigated the therapeutic effect of nobiletin on cerebral I/R injury in the t-MCAO rats. Ischemic brain damage and brain swelling were evaluated at 24 h after the start of reperfusion (Fig. 1). As assessed by 2,3,5-triphenyltetrazolium chloride (TTC) staining, nobiletin greatly reduced brain damage compared with the vehicle (Fig. 1A and B). This reduction in brain cell death by nobiletin was observed in the ischemic region. Brain swelling was calculated by the increase in the size of the right hemisphere as compared with that of the left one (Fig. 1C). One hour of ischemia and 24 h of reperfusion increased the volume of the ischemic hemisphere compared with that of the non-ischemic one. The degree of brain swelling was significantly reduced by the administration of nobiletin. Brain swelling was not observed in the sham-operated group. In addition, nobiletin treatment did not induce hemorrhage.

2.2. *Fluorescence-labeled nobiletin accumulated in the ischemic region of t-MCAO rats*

To confirm the delivery of nobiletin to the damaged region, we performed *ex vivo* fluorescence imaging of TokyoGreen-conjugated nobiletin (TG-nobiletin) injected into t-MCAO rats after reperfusion (Fig. 2). Although the fluorescence of TG-nobiletin was detected both in ischemic and non-ischemic hemispheres, a higher accumulation was observed in the ischemic region. These data suggest that nobiletin given immediately after the start of reperfusion rapidly accumulated in the ischemic region and provided a prompt therapeutic effect on I/R injury.