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Research report

Protective effects of the astaxanthin derivative, adonixanthin, on brain hemorrhagic injury

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HIGHLIGHTS

- Carotenoids are effective against hemorrhagic brain injury.
- Adonixanthin exerted protective effects against oxidative stress.
- Adonixanthin improved BBB permeability in an autologous blood injection model.

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ABSTRACT

Astaxanthin is beneficial for human health and is used as a dietary supplement. The present study was performed in order to examine the protective effects of the astaxanthin derivative, adonixanthin, against cell death caused by hemoglobin, collagenase, lipopolysaccharide, and hydrogen peroxide, which are associated with hemorrhagic brain injury.

In an *in vitro* study, adonixanthin exerted cytoprotective effects against each type of damage, and its effects were stronger than those of astaxanthin. The increased production of reactive oxygen species in human brain endothelial cells in the hemoglobin treatment group was inhibited by adonixanthin. Moreover, adonixanthin suppressed cell death in SH-SY5Y cells. In an *in vivo* study, the oral administration of adonixanthin improved blood-brain barrier hyper-permeability in an autologous blood ICH model. We herein demonstrated for the first time that adonixanthin exerted protective effects against hemorrhagic brain damage by activating antioxidant defenses, and has potential as a protectant against intracerebral hemorrhage.

1. Introduction

The main pathology of intracerebral hemorrhage (ICH) is vascular rupture, which is related to vascular fragility based on aging or hypertension (Sacco et al., 2009). Vascular leakage after rupture directly or indirectly damages brain parenchymal tissue (Garcia and Ho, 1992; Keep et al., 2012; Qureshi et al., 2009). Excessive ROS production has been strongly implicated in the exacerbation of ICH. Increased ROS production causes cell death and disruption of the blood-brain barrier (BBB) due to lipid peroxidation, DNA damage, and protein peroxidation (Qu et al., 2016). Moreover, inflammation is known to be involved in ICH-induced secondary brain injury (Feng et al., 2015).

Various animals and plants contain red or yellow pigment carotenoids. These carotenoids exhibit antioxidant, anti-inflammatory, anti-tumor, and immunomodulatory activities (Chan et al., 2009; Khan et al., 2010). Among the carotenoids contained in microalgae and seafood, such as salmon, trout, and shrimp, astaxanthin has the most prominent antioxidant (Yan et al., 2016). The antioxidant activity of

Abbreviations: NF-kB, Nuclear factor-kappa B; ROS, Reactive oxygen species; H_2O_2, Hydrogen peroxide; Nrf2, Nuclear factor erythroid 2-related factor 2; ICH, Intracerebral hemorrhage; RBC, Red blood cell; HBMVECs, Human brain microvascular endothelial cells; SH-SY5Y cells, Human brain neuroblastoma cells; LPS, Lipopolysaccharide; VE-cadherin, Vacular endothelial-cadherin; HO-1, Heme oxygenase-1; ERK, Extracellular Signal-regulated Kinase; ECM, Extracellular matrix; DMSO, Dimethylsulfoxide; DMEM, Dulbecco's Modified Eagle's medium; FBS, Fetal Bovine Serum; PBS, Phosphate buffered saline; PI, Propidium iodide; CM-H2DCFDA, 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate, acetyl ester; TBI, Traumatic brain injury

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astaxanthin was shown to be approximately 6000-fold stronger than that of vitamin C (Nishida, 2007). Therefore, astaxanthin is widely used in cosmetics and dietary supplements. Previous studies reported that astaxanthin has multiple mechanisms of action, such as the scavenging of singlet oxygen *via* conjugated double bonds, suppression of lipid peroxidation, and inhibition of nuclear factor-kappa B (NF-kB) (Jiang et al., 2016; Kishimoto et al., 2016; Zhou et al., 2017). Astaxanthin also shows high transferability to brain tissue, and protects brain vessels from cerebrovascular diseases, such as cerebral ischemia and subarachnoid hemorrhage (Pan et al., 2017; Wu et al., 2014). Astaxanthin was found to ameliorate oxidative stress, which is closely involved in the exacerbation of various diseases due to the overproduction of reactive oxygen species (ROS), the superoxide radical, hydroxyl radical, and hydrogen peroxide (H_2O_2) (Wu et al., 2002). Astaxanthin is more effective than other carotenoids, such as β-carotene, particularly in scavenging the singlet oxygen (Fukuzawa, 2000). The conjugated double bonds and terminal ring moieties of astaxanthin have been shown to trap radicals on membrane surfaces and internal membranes (Goto et al., 2001).

"Adonixanthin" and "adonirubin" are intermediate products in the generation of astaxanthin (Fig. 1A). These products also have conjugated double bonds and exhibit strong antioxidant activities, similar to astaxanthin. These products were previously shown to inhibit lipid peroxidation and quench singlet oxygen, similar to astaxanthin (Maoka et al., 2013). The suppression of lipid peroxidation and quenching of singlet oxygen also improved brain damage associated with ICH. Therefore, these intermediates may be effective against hemorrhagic brain injury; however, their effects on ICH currently remain unknown.

Collagenase is an enzyme that degrades extracellular matrix (ECM) components, such as collagen and laminin, and replicates ICH conditions with good reproducibility. A collagenase-induced ICH model is suitable for evaluating the formation of hematomas to induce vascular rupture *in vivo*. In addition, *in vitro* mono- and multi-cultured cells, such as endothelial cells, were previously shown to be damaged by collagenase (Takagi et al., 2015). Therefore, this model is appropriate for evaluating endothelial cell damage. An autologous blood model was previously shown to be suitable for assessing blood component effects (Zhu et al., 2014). The main component in red blood cells (RBCs) is hemoglobin. Under ICH conditions, RBCs within hematomas release hemoglobin into brain tissue (Katsu et al., 2010; Wang et al., 2002; Yang et al., 2013), which then damages neurons by mediating the generation of ROS (Gram et al., 2013). These findings indicate that hemoglobin is harmful to brain tissue. In these models, endothelial and neural cells were damaged *via* oxidative and inflammatory pathways.

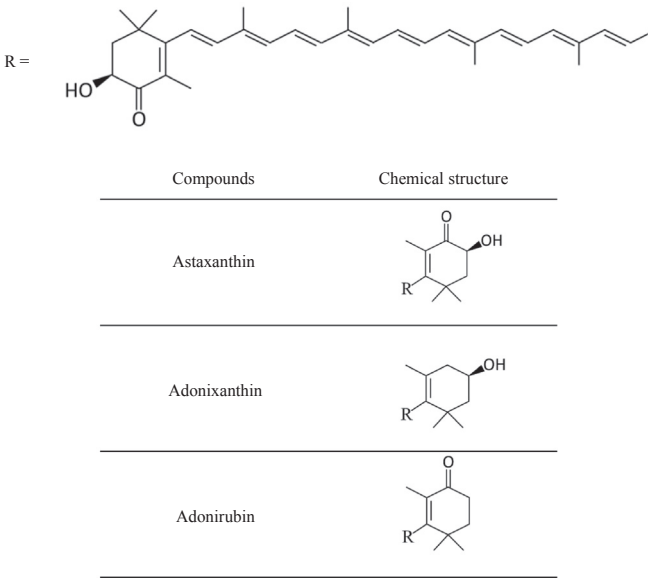

Fig. 1. Chemical structures of carotenoids. The production processes of astaxanthin, adonixanthin, and adonirubin.

The aim of the present study was to investigate the effects of these carotenoids (astaxanthin, adonixanthin, and adonirubin) on *in vitro* brain injury models derived from oxidative and inflammatory stress. We used human brain microvascular endothelial cells (HBMVECs) and human neuroblastoma cells (SH-SY5Y cells) to confirm the efficacy of adonixanthin against cell damage induced by hemoglobin, collagenase, lipopolysaccharide (LPS), and H_2O_2 *in vitro*. These models have been shown to replicate the pathology of hemorrhagic stroke (Feng et al., 2016; Hu and Liu, 2016; Lan et al., 2017). We also examined the effects of adonixanthin on vascular permeability by measuring Evans blue leakage in an autologous blood injection model. We herein discuss the potential of the intermediates of astaxanthin for ICH.

2. Results

2.1. Adonixanthin and adonirubin reduced hemoglobin-induced cell death and ROS production in HBMVECs

We evaluated hemoglobin-induced cell death as shown in Fig. 2A. Pretreatments with adonixanthin and adonirubin at 1 μM significantly decreased hemoglobin-induced endothelial cell death and ROS production (Fig. 2B and C). Astaxanthin at 1 μM slightly reduced this cell death and ROS production. Adonixanthin also dose-dependently decreased hemoglobin-induced endothelial cell death and ROS production (Fig. 2D and E).

2.2. Effects of adonixanthin on hemoglobin injury in HBMVECs

We investigated the antioxidant mechanisms of action of adonixanthin. The level of heme oxygenase-1 (HO-1), an antioxidant factor, was increased by the adonixanthin pretreatment with hemoglobin (Fig. 2G).

Vascular endothelial (VE)-cadherin is a transmembrane component of the endothelial adhesion junction. Increases in vascular vulnerability due to a compromised endothelial adhesion junction have been identified as a risk factor for the onset of ICH (Keep et al., 2014). The adonixanthin pretreatment increased VE-cadherin levels regardless of the presence of hemoglobin (Fig. 2H).

2.3. Adonixanthin dose-dependently reduced collagenase-induced cell death in HBMVECs

We investigated the protective effects of adonixanthin against collagenase injury in HBMVECs, as shown Fig. 3A. Adonixanthin significantly decreased collagenase-induced cell death (Fig. 3B and C). Other carotenoids (adonirubin and astaxanthin) also reduced collagenase-induced cell death (Supplemental Fig. 1B and C). However, astaxanthin did not significantly decrease cell death at 0.1 or 0.3 μM. In this experiment, adonixanthin at 1 μM was the most effective concentration without toxicity.

In order to elucidate the mechanisms underlying the protective effects of carotenoids against collagenase-induced damage, we focused on ERK1/2 expression. A previous study reported that the up-regulation of ERK1/2 phosphorylation caused a neuroinflammatory response in an *in vivo* collagenase-induced ICH model (Liu et al., 2016). Western blotting revealed the up-regulation of ERK1/2 phosphorylation following an exposure to collagenase. Phosphorylated ERK1/2 was significantly suppressed by adonixanthin at 1 μM (Fig. 3D).

Adonixanthin at 1 μM increased VE-cadherin levels significantly more than collagenase (Fig. 3E). Moreover, adonirubin at 1 μM suppressed pERK expression caused by the collagenase treatment and increased VE-cadherin levels (Supplemental Fig. 1D and E). These effects were similar to those of adonixanthin.